Insect Systematics & Evolution

An International Journal of Systematic Entomology

(Formerly *Entomologica scandinavica*)

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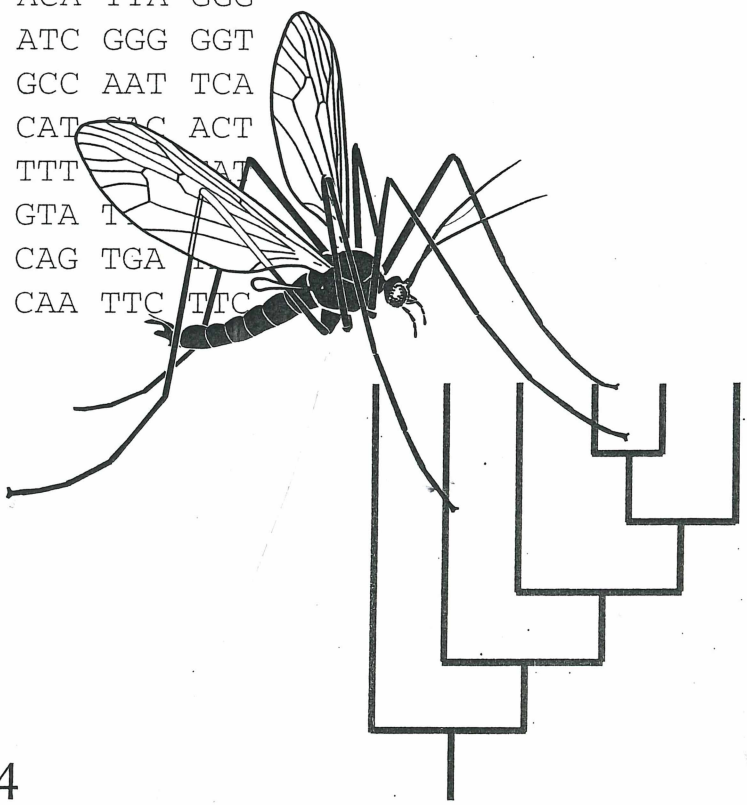

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European bumblebees (Hymenoptera: Bombini) – phylogenetic relationships inferred from DNA sequences

BO VEST PEDERSEN

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The phylogenetics of 40 taxa of European bumblebees were analysed based on PCR amplified and direct sequenced DNA from one region of the mitochondrial gene *Cytochrome Oxidase I* (1046 bp) and for 26 taxa from two regions in the nuclear gene *Elongation Factor 1α* (1056 bp). The sequences were aligned to the corresponding sequences in the honey bee. Phylogenetic analyses based on parsimony, as well as maximum likelihood, indicate that the bumblebees can be separated into several well-supported clades. Most of the terminal clades correspond very well with the clades known from former phylogenetic analyses based on morphology and recognized as the subgenera: *Mendacibombus*, *Confusibombus*, *Psithyrus*, *Thoracobombus*, *Megabombus*, *Rhodobombus*, *Kallobombus*, *Alpinobombus*, *Subterraneobombus*, *Alpigenobombus*, *Pyrobombus*, *Bombus* and *Melanobombus*. All the cuckoo bumblebees form a well-supported clade, the subgenus *Psithyrus*, within the true bumblebees. All the analyses place *Kallobombus* as the most basal taxon in contradiction to former analyses. The other deeper nodes of the phylogenetic trees, which are weakly supported, deviate significantly from former published trees - especially the trees based on *mtCO-I*. Presumably, the reasons are that multiple hits and the strong bias of the bases A and T blur the relationships in the deepest part of the trees. Analyses of the region in *mtCO-I* show a very strong A+T bias (A+T= 75%), which also indicate preferences in the use of codons with A or T in third positions. In closely related entities, there is only a weak transversion bias (A+T). In the studied regions in *EF 1-α*, no nucleotide bias is observed. The observed differences in bases between the investigated taxa are relatively small and the gene is too conserved to solve all the questions that the analyses of the deeper nodes using *mtCO-I* raise.

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Introduction

Bumblebees are coloured, hairy bees that constitute a well-defined tribus, Bombini, within the family Apidae. Most of the nearly 300 known species are from the northern hemisphere. In the southern hemisphere, the only few native species found are in the East Indies and South America. Most species are found in arctic, temperate and especially alpine environments. In the tropics, most of the few known species live in highlands. Generally, bumblebees are social insects. A hive consists of the founder, a mated queen, and no more than a few hundred unmated females, the workers. There are, of course, also cells with eggs and larvae in an established nest. The queen lays the eggs and the workers provide the larvae with food, which consists of nectar and pollen. The adults get their energy from their main food, nectar. The hive is established early in the spring and reaches a culmination in early summer when the amount of flowers is highest. In the late summer, large females, the queens for the new generation, are produced. Simultaneously, males are produced from unfertilised eggs. After mating the males die and the mated queens hibernate in a lair, commonly in the soil. The queens fly out the next year and attempt to establish a new colony, e.g. in the disused nest of a rodent. This life cycle, adapted to temperate climates with changing seasons, is common to most of the bumblebees. Only a few species from the tropics have colonies that persist for more than a year. About 45 species of cuckoo

bumblebees, which often are placed in the (sub)genus *Psithyrus*, have a different life mode. They are nest parasites. The queens usurp colonies of true bumblebees, expel the foundress queens and take control of the colonies. The indigenous workers continue as food providers for the parasitic queen and her offspring, which only develop into queens or males. Intra- as well as interspecific usurpation also seems frequent among true bumblebees. Queens unsuccessful in establishing their own colony will usurp a colony of their own species (intraspecific) and queens of a few species (*Bombus hyperboreus* and *B. inexspectatus*), which commonly lack workers, will also usurp the nests of related species (Milliron & Oliver 1966; Yarrow 1970; Richards 1973; Pape 1983).

In spite of their size and conspicuous coloration, identification of species is often difficult because several species have the same general appearance in colour and morphology, suggesting various types of mimicry (Plowright & Owen 1980; Delmas 1981). Often, only small differences in morphology seem to distinguish the species. The greatest interspecific variation is found in the structure of the male genitalia. However, collected material can be very difficult to identify because males are only found during a short period in the late summer and many closely related species don't vary even in the male genitalia. The apparent lack of interspecific variation in morphology is presumably due to the fact that pheromones play a major role in communication among bumblebees. The use of genetic markers, allozymes detected by electrophoresis or DNA sequences, have, on the other hand, shown to be useful tools in delineating entities, e.g. populations, subspecies and species.

The tribus Bombini seems to be a well-defined clade of long tongued, densely hairy, social bees closely related to the honey bees, Apini, the orchid bees, Euglossini, and the stingless bees, Meliponini. Cladistic analyses based on morphology place the Euglossini as the sister group of Bombini (Winston & Michener 1977; Kimsey 1984; Michener 1990), whereas analyses based on mitochondrial and nuclear DNA sequences place the Meliponini, as the sister group of Bombini (Cameron 1991 1993; Koulianos et al. 1999; Cameron & Mardulyn 2001). In the last mentioned study, discordance between morphological and molecular based phylogenies was observed.

The species of *Bombus* have been grouped in several subgenera on basis of morphology, especially structures in the male genitalia (Richards 1968; Williams 1985 1991 1994). The cuckoo bumblebees have, in addition to their special way of life, some proper morphological features that seem to be correlated with their parasitic life. Several phenetic, evolutionary, and cladistic analyses based on morphology, protein electrophoretic data, and mtDNA data indicate monophyly of the cuckoo bumblebees (Milliron 1971; Williams 1985, 1991, 1994; Plowright & Stephen 1973; Pekkarinen et al. 1979; Obrecht & Scholl 1981 (except *P. rupestris*); Pamilo et al. 1981, 1987; Ito & Sakagami 1985; Pedersen 1996). However, the relationships suggested for cuckoo bumblebees to the different groups of species of true bumblebees differ from author to author. A polyphyletic origin of the cuckoo bumblebees has been proposed based on morphology (Richards 1927; Tkalcû 1972) and on chemotaxonomic analyses (Bellés et al. 1987).

The relationships of the different groups of bumblebees have been analysed and although several different phylogenetic hypothesis have been proposed there seems to be some congruence in the groupings of species. Studies based on morphological features (Milliron 1971) and cladistic analyses based on morphology (Williams 1985, 1991, 1994) differ considerably, with Milliron (1971) arguing for a polyphyletic origin of the bumblebees. Phenetic analyses based upon protein electrophoretic data (Pamilo et al. 1981 and 1987; Obrecht & Scholl 1981; Pekkarinen 1979; Pekkarinen et al. 1979), chemotaxonomic data (Bellés et al. 1987), morphological features of the wing (Plowright & Stephen 1973), and a comprehensive phenetic analysis (Ito 1985) based on the male genitalia from 38 of the 42 so far proposed subgenera differ as well. Williams (1985 1991, 1994), in his cladistic analyses based on morphological characters in male genitalia, places the '*mendax*' group as the most basal bumblebees and the rest of the true bumblebees as the sister group to the cuckoo bumblebees. Most other analyses based on morphology, electrophoretic enzyme data as well as mtDNA data place the cuckoo bumblebees in a more distal position of the tree (Pamilo et al. 1981; Ito 1985; Pedersen 1996). A basal position of the '*mendax*' branch was also proposed by Ito (1985). Various aspects of the phylogeny of bumblebees have been studied through analyses of mitochondrial DNA sequences from the genes *Cytochrome Oxidase I (CO-I)* and *II (CO-II)* and *Cytochrome b*

(Cyt b) (Pedersen 1996; Koulianos 1999; Koulianos & Schmid-Hempel 2000). Among other things, the subgenera *Pyrobombus* and *Melanobombus*, do not reflect monophyletic groups in these studies.

The present study is an attempt to analyse, by help of DNA data, the phylogenetic relationships of a collection of 40 taxa of cuckoo bumblebees and true bumblebees collected from different parts of Europe and Greenland. Two DNA regions were sequenced and analysed. A segment of 1056 bp in the relatively conserved mitochondrial gene *CO-I* was used to delimit the investigated taxa and to analyse their groupings. A region of 1153 bp from two exons interrupted by an excluded intron in the conserved nuclear gene *EF-1α* was also sequenced and analysed in 26 taxa in order to study the most basal part of the phylogenetic tree of the bumblebees. The two genes were chosen because *CO-I* has been thoroughly analysed in several insect studies and has shown its usefulness in delimiting groups and in phylogenetic analyses. *EF-1α* has been used successfully in phylogenetic analyses of the deeper nodes within several orders of insects (Cho et al. 1995; Danforth et al. 1999; Reed & Sperling 1999).

Materials and methods

Bumblebees and cuckoo bumblebees from various parts of Europe and Greenland were collected for the DNA sequencings. The specimens were frozen immediately after collection and either stored at -80°C or preserved in 92-96% ethanol. Tab. 1 lists the studied material. Identification of species was aided by descriptions and keys in Alford (1975), Amiet (1996), Bertsch (1997), Erlandsson (1979), Delmas (1976), Faester (1959), Løken (1973 1984), Milliron (1973), Prys-Jones & Corbet (1991), Rasmont (1984) and Svensson (1979).

To prevent contamination from symbionts, etc., only the thoracic musculature was used for DNA extraction. A hole was cut in the thoracic pleuron, a muscle lump removed, transferred to a microtube and frozen to -80°C. Scissors and needles used for the dissections were carefully cleaned between dissections.

Total genomic DNA was extracted following two procedures. The first procedure was replaced mid-way through the study by a second procedure, modified after Doyle & Doyle (1987), which produced better template DNA for PCR.

(1) The muscle lump was homogenized with a pestle in an Eppendorf tube with 300µl extraction buffer (100nM EDTA, 10 mM TrisHCl, 0.1 M NaCl), 30µl 10% SDS, and 7µl Proteinase K (10 mg/ml stock solution). The sample was then incubated at 60°C and gently mixed with a rotator for 1 h. DNA was first extracted with phenol and chloroform and then ethanol-precipitated following standard protocols (Sambrook et al. 1989; Hillis et al. 1996). Finally, the DNA was suspended in 30µl 0.1x TE and stored at -20°C.

(2) The muscle lump was homogenized with a pestle in a 1.5-ml Eppendorf tube with 300µl 2X CTAB extraction buffer (100mM TrisHCl, pH 8.0, 1.4 M NaCl, 20 mM EDTA, 0.2% β-mercaptoethanol (v : v), and 2% CTAB (v : v)) preheated to 60°C. Samples were incubated at 60°C and gently mixed with a rotator for 30 min. After cooling to room temperature, samples were extracted with 300µl chloroform: isoamyl alcohol (24 : 1) and the DNA precipitated with 200µl isopropanol. The final DNA preparations were stored as in the first procedure.

A nucleotide sequence of 1056 bp in *mtCO-I* was determined for 231 individuals comprising 40 taxa of bumblebees. A segment of 1500-1750 bp in the nuclear gene *EF-1α* was also sequenced in 30 individuals of 26 taxa of bumblebees. The segment in *EF-1α* consists of two regions in two exons and an intervening intron, which has a length in bases differing from species to species.

The *mtCO-I* DNA sequences were determined by direct sequencing of asymmetrically amplified (PCR) products (Gyllensten & Ehrlich 1988; Saiki et al. 1988). PCR amplifications were performed in 50-µl volumes of 1-50 ng of template DNA, 67 nM Tris-HCl (pH 8.8), 2mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM α-mercaptoethanol, 1mM each dNTP, 1 µM each primer, and 1 unit *Taq* DNA polymerase. The polymerase was added after a one min. initial denaturation at 94°C (hot start). Cycle conditions were as follows: denaturation at 94°C for 1 min., annealing at 37-50°C for 1 min. and extension at 72°C for 4 min., 30 cycles total. Amplified products were separated on agarose gels. A gel plug containing the desired fragment was transferred to a tube with 1 ml 0.1 x TE. The single-stranded DNA amplifications were performed using 1µl double-stranded amplification product as template and with the same reagents as for the double-stranded amplifications, except for the primer concentrations. The concentration of one of the primers was reduced 100-fold. The following amplification procedures were used: hot start, denaturation at 94°C for 1 min., annealing at 45-55°C for 1 min. and extension at 72°C for 2 min., 35 cycles total. The amplified

Table 1. List of investigated bumblebees with collection data: Locality, country, number of studied specimens, sex and date of collection. mtCO-I from all listed specimens have been analysed. * means species and specimens which also have been used in the EF-I-α analyses. The order and grouping in subgenera follows Williams (1994).

**Bombus (Mendacibombus) mendax* Gerstäcker, 1869		
*Hochtor (A)	1♀	1991-08-18
*Julierpass (CH)	1♀	1993-06-29
**Bombus (Confusibombus) confusus* Schenck, 1859		
*Argentat (F)	1♂	1997-07-14
**Bombus (Psithyrus) barbutellus* (Kirby, 1802)		
*Ekkodalen (DK)	1♂	1989-08-11
Tåstrup (DK)	1♂, 1♀	1989-08-11
Tüchersfeld (D)	1♂	1993-07-02
**Bombus (Psithyrus) bohemicus* (Seidl, 1837)		
*Ekkodalen (DK)	2♂, 1♀	1989-08-11
Tåstrup (DK)	1♀	1989-08-17
Tuse Næs (DK)	1♀	1990-04-22
Kaprun (A)	2♂	1993-06-30
Tüchersfeld (D)	1♂	1993-07-02
**Bombus (Psithyrus) campestris* (Panzer, 1801)		
Sorø (DK)	1♂	1995-08-20
*Edinburgh (GB)	1♂	1995-09-05
**Bombus (Psithyrus) flavidus* (Eversmann, 1852)		
*Dagali (N)	1♀	1994-07-02
Bombus (Psithyrus) norvegicus Sparre-Schneider, 1918		
Tuse Næs (DK)	1♀	1990-04-22
Bombus (Psithyrus) quadricolor (Lepeletier, 1832)		
Hochter (A)	1♀	1991-08-18
**Bombus (Psithyrus) rupestris* (Fabricius, 1793)		
Tåstrup (DK)	1♂	1989-08-11
*Tuse Næs (DK)	1♀	1990-06-10
Særløse Overdrev (DK)	1♂	1990-07-29
Munkholm Broen (DK)	1♂	1990-07-28
Gadmen (CH)	1♀	1993-06-27
Bombus (Psithyrus) sylvestris (Lepeletier, 1832)		
Ekkodalen (DK)	1♂	1989-08-11
Brorfelde (DK)	1♂	1990-07-22
Tüchersfeld (D)	1♂, 2♀	1993-07-02
Chur (CH)	1♂	1993-06-28
Kaprun (A)	3♂	1993-06-30
Mörkedalen (N)	1♀	1994-07-02
Bombus (Psithyrus) vestalis (Geoffroy, 1785)		
Ekkodalen (DK)	1♂	1989-08-11
Særløse Overdrev (DK)	1♂	1990-07-29
Holbæk (DK)	1♂	1994-07-24
Røsnæs (DK)	1♂	1996-08-25
Bombus (Thoracobombus) humilis Illiger, 1806		
Bibio (CH)	1♀	1993-06-29
Bombus (Thoracobombus) muscorum (Fabricius, 1793)		
Raghammer Odde (DK)	2♀	1997-08-12
Öland (S)	1♂	1996-08-21
**Bombus (Thoracobombus) pascuorum* (Scopoli, 1763)		
Pårup (DK)	1♂, 3♀	1992-07-24
Tuse Næs (DK)	1♀	1990-06-10
Faxe-Ladeplads (DK)	3♀	1991-07-28
Gundestrup (DK)	1♀	1994-07-27
Sorø (DK)	2♀	1995-08-20
Tuse Næs (DK)	1♂	1995-08-27
Røsnæs (DK)	2♀	1996-08-25
*Holbæk (DK)	2♂, 2♀	1996-08-25
Hven (S)	1♀	1990-06-12
Tüchersfeld (D)	2♀	1993-07-02
Einsideln (CH)	2♀	1993-06-28
*Kaprun (A)	1♀	1993-06-30
Prats de Mollo (F, Py)	2♀	1993-06-28
Argentat (F)	2♀	1997-07-14
Rondane (N)	1♀	1994-06-30
Edinburgh (GB)	1♀	1995-09-05
Crianlache (GB)	1♀	1995-09-06
**Bombus (Thoracobombus) ruderarius* (Müller, 1776)		
Bivio (CH)	3♀	1993-06-29
Tuse Næs (DK)	1♀	1990-06-10
*Kowary (P)	2♀	1997-07-02
Prats de Mollo (F, Py)	2♀	1993-08-25
Bombus (Thoracobombus) sylvarum (L., 1761)		
Sose (DK)	1♂, 2♀	1992-08-10
Øland (S)	6♀	1996-08-20/21
**Bombus (Megabombus) consobrinus* Dahlbom, 1832		
*Lom (N)	1♀	1994-07-01
Fortun (N)	1♀	1994-07-01
**Bombus (Megabombus) hortorum* (Linnaeus, 1761)		
Holbæk (DK)	1♀	1994-08-28
*Tüchersfeld (D)	3♂, 2♀	1993-07-02
Prats de Mollo (F, Py)	1♀	1993-08-25
Fortun (N)	1♀	1994-07-01
**Bombus (Rhodobombus) mesomelas* Gerstäcker, 1869		
Julierpass (CH)	1♀	1993-06-29
*Prats de Mollo (F, Py)	3♀	1993-08-25
**Bombus (Kallobombus) soroeensis* (Fabricius, 1777)		
Subspecies *soroeensis:*		
Ekkodalen (DK)	1♂	1989-08-11
Munkholmbroen (DK)	1♂	1990-07-28
*Raghammer Odde (DK)	2♀	1997-08-12
Holbæk (DK)	2♀	1990-05-28
Edinburgh (GB)	1♀	1995-09-05
Ringebu (N)	3♀	1997-08-02
Subspecies *proteus:*		
Route des Crétes,		
Grand Ballon (F)	1♀	1995-07-17
Fusch (A)	1♂	1991-08-18
Sölker Pas (A)	2♀	1993-07-01
Bivio (CH)	1♀	1993-06-29
Färnigen (CH)	3♀	1993-06-27
**Bombus (Alpinobombus) balteatus* Dahlbom, 1832		
Buskerud (N)	1♀	1994-07-03
*Dovrefjell (N)	2♀	1997-08-01
**Bombus (Alpinobombus) hyperboreus* Schönherr, 1809		
Ameralikfjorden,		
*Nuuk (Greenl)	3♀	1994-06-22/24
Ameralikfjorden,		
Nuuk (Greenl)	1♀	1994-07-10
Dovrefjell (N)	1♂	1997-08-01
Bombus (Alpinobombus) polaris Curtis, 1835		
Ameralikfjorden,		
Nuuk (Greenl)	1♀	1994-06-29
**Bombus (Subterraneobombus) subterraneus* (L., 1758)		
Allindelille Fredskov (DK)	1♀	1997-05-23
*Neubrandenburg (D)	1♀	1997-07-04
**Bombus (Alpigenobombus) wurfleini* Radoszkowski, 1859		
Färnigen (CH)	2♀	1993-06-27

Julierpass (CH)	1♀	1993-06-29
*Lenzerheide (CH)	1♀	1993-06-28
Sattelegg (CH)	1♀	1993-06-28
Kaprun (A)	1♀	1993-06-30
Prats de Mollo (F, Py)	1♀	1993-08-25
Mörkedalen (N)	1♀	1994-07-02
Bröstrud (N)	1♀	1994-07-03
*Bombus (Pyrobombus) hypnorum (Linnaeus, 1758)		
*Ekkodalen (DK)	1♂, 1♀	1989-08-11
Ekkodalen (DK)	1♂	1992-08-08
Tåstrup (DK)	1♂	1989-07-17
Copenhagen (DK)	1♀	1990-07-31
Kaprun (A)	1♀	1993-06-30
Rondane (N)	1♀	1994-06-30
*Bombus (Pyrobombus) jonellus (Kirby, 1802)		
*Rondane (N)	2♀	1994-06-30
*Bombus (Pyrobombus) monticola Smith, 1849		
Dombås (N)	1♀	1994-07-01
Dagali (N)	1♀	1994-07-02
*Ål (N)	1♀	1994-07-02
Lesjaskog, Dombås (N)	4♀	1997-08-01
Toftsetra, Dombås (N)	2♀	1997-08-01
Julierpass (CH)	4♀	1993-06-29
Chur (CH)	1♀	1993-06-28
*Sölker Pas (A)	1♀	1993-07-01
*Bombus (Pyrobombus) pratorum (Linnaeus, 1761)		
Ekkodalen (DK)	1♂	1989-08-11
Ekkodalen (DK)	4♀	1991-08-08
*Bogø (DK)	1♀	1990-04-28
Holbæk (DK)	1♀	1990-04-30
Særløse Overdrev (DK)	1♀	1990-07-29
Sorthat (DK)	2♀	1992-08-09
Svinemosen (DK)	1♀	1992-08-09
Alindelille Fredsskov (DK)	1♀	1997-05-13
Bivio (CH)	1♀	1993-06-29
Sattelegg (CH)	1♀	1993-06-28
Kaprun (A)	1♀	1993-06-30
Tückerfeld (D)	1♀	1993-07-02
Rondane (N)	1♀	1994-06-30
*Bombus (Pyrobombus) pyrenaeus Pérez, 1879		
*Färnigen (CH)	1♀	1993-06-27
Julierpass (CH)	1♀	1993-06-29
Sölker Pas (A)	1♀	1993-07-01
Bombus (Bombus) cryptarum (Fabricius, 1775)		
Ekkodalen (DK)	1♀	1992-08-08
Kaprun (A)	1♂	1993-06-30
*Bombus (Bombus) lucorum (Linnaeus, 1761)		
Tåstrup (DK)	2♂	1989-08-17
Særløse Overdrev (DK)	1♂	1990-07-29
Hven (S)	1♂	1990-06-12
Färnigen (CH)	1♀	1993-06-27
*Bivio (CH)	1♀	1993-06-29
Sattelegg (CH)	2♀	1993-06-28
Kaprun (A)	1♂	1993-06-30
Ål (N)	1♀	1994-07-02
Bröstrud (N)	1♀	1994-07-03
Skute (N)	1♀	1994-07-03
Edinburgh (GB)	2♀	1995-09-05
Bombus (Bombus) maderensis Erlandsson, 1979		
Ponta de S. Lourenco (Mad.)	1♀	1994-03-15
Bombus (Bombus) magnus Vogt, 1911		
Sölker Pas (A)	2♀	1993-07-01
Gadmen (CH)	1♀	1993-06-27
Bivio (CH)	1♀	1993-06-29
Julierpas (CH)	1♀	1993-06-29
*Bombus (Bombus) sporadicus Nylander, 1848		
*Skute (N)	2♀	1994-07-03
*Bombus (Bombus) terrestris (Linnaeus, 1758)		
Tåstrup (DK)	1♂	1989-08-22
Tåstrup (DK)	1♂	1989-09-07
*Hørsholm (DK)	1♀	1990-03-30
Tuse Næs (DK)	2♂	1990-08-30
Holbæk (DK)	2♀	1990-06-09/10
*Bølshavn (DK)	1♂, 2♀	1991-08-09
*Sose (DK)	2♀	1991-08-10
Edinburgh (GB)	2♀	1995-09-05
Bombus (Bombus) sp. A		
Böverdalen (N)	1♀	1994-07-01
*Bombus (Melanobombus) lapidarius (Linnaeus, 1758)		
Brorfelde (DK)	1♀	1989-08-14
*Ulfshale (DK)	1♀	1990-04-28
Holbæk (DK)	1♀	1990-05-28
Sose (DK)	1♂	1992-08-10
Raghammer Odde (DK)	2♀	1992-08-06
Sorthat (DK)	3♀	1992-08-09
Ål (N)	1♀	1994-07-02
Bombus (Melanobombus) sichelii Radoszkowski, 1859		
Kaprun (A)	1♀	1993-06-30
Gadmen (CH)	1♀	1993-06-27

DNA was strained off using 30,000 NMWL filter units (Millipore) and finally suspended in 20 µl ddH_2O. The aforementioned procedure was done twice for each template reducing each primer once. Both strands of the amplified region were sequenced directly (Sanger et al. 1977) using the Sequenase sequencing kit 2.0 (US Biochemical Corp.).

Different combinations of primers (see Tab. 2) were used for the single-strand amplification. In most of the amplifications, the primer products based on AP-L-1954/1960 and AP-H-3081/3103 were used as template for the single-stranded amplifications. This procedure gave rise to short and legible sequences and ensured overlap of sequences for confirmation.

The nucleotide sequence of the 1000-1400 bp segments in *EF-1α* was determined using slightly different procedures. The double-stranded amplifications were analogous to the mentioned procedures except for the primers. Elobom-L-0504/0540 and Elobom-H-1853 (see Tab. 3) were used for the amplifications of a large segment, which was used as the template for the cyclical single-stranded amplification. The PCR double-stranded products were filtered using Qiagen

Table 2. Oligonucleotide primers for PCR amplification and sequencing of a region in *mtCO-I*.

Designation	Sequence	Location
AP-L-1954	5′-agcaatgatcaaatttataatac-3′	(1931..1954)
AP-L-1960	5′-tgatcaaatttataatacaattgt-3′	(1937..1960)
AP-L-2013	5′-tatagttatacctattttaattg-3′	(1991..2013)
AP-L-2176	5′-actggatgaacagtatatcc-3′	(2157..2176)
AP-L-2266	5′-ggaatttcttccattattgg-3′	(2247..2266)
AP-L-2504	5′-caacatttattttgatttttgg-3′	(2481..2503)
AP-L-2529	5′-tccagaagtttatatttttaattc-3′	(2508..2529)
AP-L-2532	5′-tccagaagtttatatttttaattttac-3′	(2508..2532)
AP-L-2776	5′-tgattagcaacatatcatggttc-3′	(2754..2776)
AP-H-2343	5′-actgtaatacaaactgatca-3′	(2362..2343)
AP-H-2551	5′-cctctttcatttattacaatatgag-3′	(2575..2551)
Ap-H-2601	5′-aattcctaatattgcataaattattct-3′	(2627..2601)
AP-H-2650	5′-tccgactgtaaatatgtgatgtgctc-3′	(2675..2650)
AP-H-2836	5′-taatattactccagttagtcctccaa-3′	(2861..2836)
AP-H-2890	5′-tccaacaacgtaatatgtatcat-3′	(3012..2990)
AP-H-2930	5′-gcaaatacagctcctattgat-3′	(2950..2930)
AP-H-3082	5′-tcgtggtatagatattagtc-3′	(3101..3082)
Ap-H-3100	5′-tggatagtctgaataacgtc-3′	(3119..3100)
AP-H-3103	5′-aatctggatagtctgaataac-3′	(3123..3103)

Note. Location indicates the position according to Crozier & Crozier (1993) for the 3′and 5′ ends of the primers. L and H in the primer designation indicate light and heavy strands.

Quick-Spin Columns. Afterwards, 3µl double-stranded amplification product was used as template for the cyclical single-stranded amplifications under the following conditions: the reactions were performed in 10 µl volumes of 4 µl readymix, 1.8 µl ddH_2O, 1.2 µl primer and 3 µl template; denaturation at 96°C for 10 sec., annealing for 5 sec. at the same temperature as used under the double-stranded amplification, and extension at 60°C for 4 min., 25 cycles total. Different primers (see Tab. 3) were used to get overlapping sequences and to get amplification products of the sense and antisense strands. The single-stranded amplified products were cleaned using Centrisep columns or ethanol precipitation and dried. Afterwards, the product was resuspended and electrophoresed on an Applied Biosystems international 377 automated sequencer using the recommended standard procedures.

The *mtCO-I* data were aligned optically and compared with the *CO-I* sequence published for *Apis mellifera ligustica* (Crozier & Crozier 1993). The *EF-1α* sequences were aligned and compared with the corresponding F1 sequence (Danforth & Ji 1998) in *Apis mellifera* (GenBank, Acc. No. X52884) (Walldorf & Hovemann 1990) using the program Sequencher 3.0 (Gene Codes Corporation, Inc. Ann Arbor, Michigan).

In order to avoid contamination of the mitochondrial PCR-amplified products by nuclear copies (Numts) (Bensasson et al. 2001), only tissue rich in mitochondria (flight-muscles) was used for the DNA extractions, large fragments were used for the double-stranded amplifications, and only amplified products of correct lengths without ghost bands were used in the subsequent procedures. All the mtDNA sequences were tested for stop codons and none were found.

Phylogenetic analyses under parsimony and maximum likelihood were carried out using PAUP 4.0b1&2 (Swofford 1998) in combination with MacClade 3.05 (Maddison & Maddison 1992) and PHYLIP 3.5c/3.6 (Felsenstein 1993 & 2000). Maximum likelihood was performed with the HKY85 (Hasegawa et al. 1985) model for nucleotide substitutions. Different transition/transversion ratios and different ratios in the discrete gamma distribution (α) accounting for the rate variation among sites were used for optimising the Ln-likelihood value. To evaluate the strength of the internal branches in the trees based on parsimony, the bootstrap procedure in PAUP was used, in addition to the 'decay index' (Bremer 1994) calculated by PAUP and the program TreeRot v. 2 (Sorenson 1999). The best of the resulting trees were then challenged by alternative trees devel-

Table 3. Oligonucleotide Primers for PCR Amplification and Sequencing of a region in *Elongation Factor 1α*.

Designation	Sequence	Location
Elobom-L-0501*	5′-atcgaaaagttcgagaaggaggc-3′	(0479..0501)
Elobom-L-0540*	5′-tcgttcaagtacgcctgggt-3′	(0521..0540)
Elobom-L-0570	5′-gacaagttgaaagcagaacgtgaacg-3′	(0545..0570)
Elobom-L-0747	5′-gaattcgaagctggtatctc-3′	(0727..0747)
Elobom-L-1062	5′-accctgatcgaagcccttga-3′	(1043..1062)
Elobom-L-1176	5′-gtcggtctggtagagaccggtat-3′	(1154..1176)
Elobom-L-1472*	5′-tcggtcgagatgcatcacg-3′	(1454..1472)
Elobom-H-0812*	5′-tccatcttgttcactccaac-3′	(0831..0812)
Elobom-H-1116	5′-accgatcttgtacacgtcct-3′	(1135..1116)
Elobom-H-1524	5′-tctcaactctttcacggagatgt-3′	(1546..1524)
Elobom-H-1527	5′-tctcaactctttcacggaga-3′	(1546..1527)
Elobom-H-1667*	5′-atatgagcggtgtgacaatc-3′	(1667..1686)
Elobom-H-1853*	5′-tgacgcatgtcgcgaaccgcgaa-3′	(1875..1853)

Note. * Primers modified from Regier & Shultz (1997). Location indicates the position according to Walldorf & Hovemann (1990) for the 3′and 5′ends of the primers. L and H in the designation indicate light and heavy strands.

oped using the User Tree options in DNApars, DNAML (PHYLIP 3.5c) and the program DAMBE (Xia 2000). In all the analyses, *Apis mellifera* was used as the outgroup.

The sequence data for all the species of bumblebees and cuckoo bumblebees in this paper have been submitted to the GenBank Data Libraries under the following Accession Nos.: *mtCO-I*, AY18097-AY181194; *EF-1α*, AY180348-AY 180373.

Species identity problems

As documented in several papers (Krüger 1928, 1931, 1940, 1951, 1954, 1956, 1958; Reinig 1981; Pekkarinen 1979), many species of bumblebees can be very difficult to identify because they vary in colour, size and morphology. The subgenus *Bombus* (= *terrestribombus*-group) with the species *B. terrestris*, *B. lucorum*, *B. cryptarum*, *B. magnus*, *B. maderensis* and *B. sporadicus* vary so much that only 'typical' specimens can be identified. In spite of considerable intraspecific variation, several studies based on morphology (Krüger 1951, 1954, 1956, 1958; Pekkarinen 1979; Rasmont 1984; Rasmont et al. 1986), crossing experiments (de Jonghe & Rasmont 1983), enzyme electrophoretic data (Scholl & Obrecht 1983; Pamilo et al. 1984), enzyme mobilities, analyses of pheromones (Pamilo et al. 1996) and analyses of the compounds of the secretions from male labial glands (Bertsch 1997) have proved the existence of the species *B. terrestris*, *B. lucorum*, *B. magnus*, *B. cryptarum* and *B. sporadicus*. Comparisons of DNA sequences (*mtCO-I*) from these species in the present study show that they can be characterized on base differences (Tab. 5 and 8), but identification based on morphology is still problematic. *B. terrestris*, *B. lucorum*, *B. magnus* and *B. cryptarum* live sympatrically in the central to northern part of Europe. The species designated *Bombus sp. A* looks like a light-coloured *B. lucorum*, but differs so much in DNA that it must have its own independent species position. The collected material of *B. hortorum* contains a few specimens in which the shape of the yellow bands on thorax is identical to the species *B. ruderatus*, but the entire collection has identical DNA sequences and is therefore treated as *B. hortorum*. The collection of *B. pyrenaeus* contains specimens from Austria with coloration like *B. brodmannicus* (Tkalcû 1973), but with DNA sequences like *B. pyrenaeus* and is therefore treated as *B. pyrenaeus*. *B. soroeensis* is divided into two subspecies: *B. s. soroeensis* and *B. s. proteus*. *Bombus s. soroeensis* is distributed in western Europe (including Great Britain) and northern and eastern Europe. Typical specimens are black with a yellow band on the front of the thorax, an often interrupted yellow band on the front of abdomen and white terminal abdominal segments, though several melanistic specimens have been observed. *Bombus s. proteus*, distributed in a triangular area starting in the Alps and stretching northward to Zealand in Denmark (Löken 1973; Reinig 1939), differs by being all black with red terminal abdominal segments. In

Table 4. Polymorphisms in a segment of the 1056 bp in *mtCO-I* in European bumblebees and cuckoo bumblebees. 1st pos. x, 2nd pos. x, 3rd pos. x. Position 1 corresponds to position 2029 in Crozier & Crozier (1993).

Species	Locality	Variable sites	Position
B. mendax:	A CH	a a a a a a t a c a t g t t t t a t t t	450,831,834,876,906,909,912, 921,977,1038
B. (P) barbutellus	D DK	t c	321
B. (P.) campestris	DK GB	a t t c a c	284,286,489
B.(P.) bohemicus	A A + D DK	t a a g a a	 537,714
B.(P.) rupestris	CH DK	t c	 819
B. (P.) sylvestris	A + CH D DK + N	t t c t c t c t c	 171,483,1044
B. muscorum	DK DK S	t t t t c g a a a t t t a a t	 672,740,756,834,903
B. pascuorum	A + CH + F D DK DK GB N	g a t c a a g a t c a t g a t c a a g t a c a a a a t c a a a a t t g a	 193,213,684,972,993,1014
B. ruderarius	CH + P + DK F	g a	 522
B. sylvarum	DK + S S	t a t a t a c c t a g a t t	 528,555,598,609,684,714,948
B. hortorum	F + D + DK N	c t t c	 510,741
B. mesomelas	CH F	g a	 706
B. soroeensis	A + CH CH + DK DK GB	a g t a t c a g t a t t a a a a t t g g t t c t	 522,654,840,942,1011,1044
B. hyperboreus	N Grnl. Grnl.	t g a t t t a g t t t t g t t c a a t c t t a a c t t t a t t t a a t t c c g a c c c c a a a t	97,138,192,210,250,453,462, 513,522,624,779,783,807,834, 906,907
B. subterraneus	D DK	a g a g c g a g a t	 148,264,573,960,1008
B. wurfleini	A + CH F N	g a a t t t a c g c c c g a a t t c	 141,156,193,213,961,1041
B. hypnorum	A + DK N	a g	345
B. monticola	A + CH N N	t a a c c g c c c g c t	 10,489,531,858
B. lucorum	A + CH DK N (GB*)	t t t c c c	 957,994
B. terrestris	DK DK GB	t t t t a t t c a c c c t t t t t t t a t t c a c c c a c t c c c c t c c t t t t t t t c	19,103,318,333,342,370,391, 429,432,447,453,483,930,957, 977

Note. *B. lucorum* (GB*) is omitted from the table and treated as an equal taxon in the analyses.

spite of some observed geographical differences in the DNA sequences the two morphotypes clearly belong to one species. *B. pascuorum* is a highly variable species and numerous analyses of the variation have been published (Krüger 1928, 1931; Reinig 1970; de Ruijter & Wiebes 1975; Pekkarinen 1979). The species has been divided into several subspecies based on colour differences. Melanistic forms have been observed over nearly the entire distribution range. In contrast to the morphological variation, Pamilo et al. (1984) did not find a corresponding geographical genetic variation in an electrophoretic study of allozyme mobilities. Based on mtDNA data (*Cyt b*), Pirounakis et al. (1998) found small differences in haplotypes of *B. pascuorum* from areas north and south of the Alps. This corresponds well with the observations of this study. Small geographical differences do not alter the species position of *B. pascuorum*

Results

Cytochrome Oxidase I

Sequencing of the *mtCO-I* region shows that several of the species vary in base compositions when comparing specimens from different localities (see Tab. 4). Only a few of the species sequenced from several localities seem to be monomorphic, no variation was found in *B. pratorum*, *B. pyrenaeus* and *B. lapidarius*. Species with polymorphic sites differ commonly in transitions; in Tab. 4, only *B. mendax* and *B. sylvarum* have more transversions than transitions. The 13 species with 1-5 polymorphic sites have, on average, an intraspecific transition/transversion ratio of nearly 3 as opposed to the general transition/transversion ratio of 0.45 for the 40 taxa used in the phylogenetic analyses. The species *B. soroeensis*, with the two subspecies *s. soroeensis* and *s. proteus*, is remarkable because the English specimen (*B. s. soroeensis*) differs from the Continental specimens more than the two haplotypes of Danish *B. s. soroeensis* differ from the central European specimens of *B. s. proteus*. In the studied material of *B. pascuorum*, 6 genotypes with 6 polymorphic sites were observed. These 6 mtDNA genotypes do not agree with colour-based division of this species into subspecies.

Four of the species are considerably more polymorphic than the rest. The two specimens of *B. mendax* from Austria and Switzerland differ in 10 sites, of which 8 are transversions (A↔T) in third positions. Two are transitions, one (C↔T) in a second position of a codon and one (G↔A) in a third

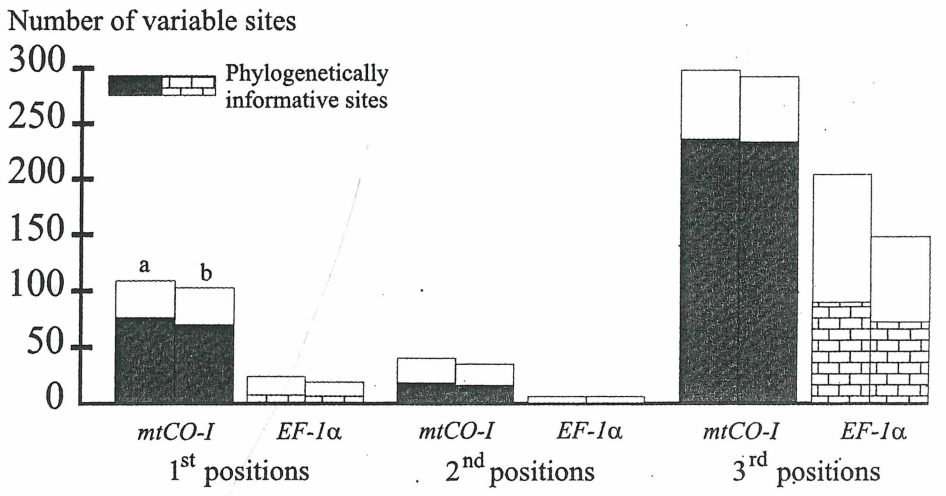

Figure 1. Distribution of variable sites and phylogenetically informative sites for 1st, 2nd and 3rd positions in *mtCO-I* (1056 bp) and *EF-1α* (1153bp). *mtCO-I*: (a) The 40 bumblebee taxa plus the honey bee. (b) The 40 bumblebees. *EF-1α*: (a) The 26 bumblebee taxa plus the honey bee. (b) The 26 bumblebees.

Table 5. Matrix of Observed Substitutions for First + Second Positions (below diagonal) and Third Positions (above) in the *CO-I* codons

	Means	SD	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	Total
			96,7	79,1	71,6	81,5	75,6	84,9	71,2	68,6	78,1	80,6	83,6	78,0	75,0	70,6	78,1	76,8	77,4	76,4	75,7	71,2	69,2	75,9	73,5	81,0	71,5	76,6	101,7	74,0	73,0	69,9	72,8	80,7	79,6	87,8	77,7	95,2	88,6	78,2	71,7	82,7	87,8	78,5
			8,7	14,9	11,0	12,0	20,6	13,0	16,5	10,9	11,2	12,7	12,0	15,7	20,7	18,9	18,3	19,0	10,9	9,9	13,2	15,6	16,2	12,2	13,9	12,2	18,1	9,8	6,1	10,7	11,8	16,7	17,5	9,3	9,7	11,6	10,3	17,3	16,1	14,5	9,5	14,3	13,5	15,7
1 A. mellifera	50,3	4,5		91	86	103	106	108	85	85	97	99	108	106	106	95	103	105	94	91	104	72	84	98	97	102	85	97	110	97	95	84	97	91	96	102	86	110	102	94	91	99	105	
2 Balteatus	25,9	10,2	47		67	84	82	82	60	35	72	80	86	85	80	78	80	85	79	81	80	64	63	56	77	78	63	84	115	79	60	52	76	82	87	92	88	107	98	92	76	93	96	
3 Confusus	31,6	8,1	47	28		70	72	71	63	64	71	70	72	73	72	61	74	69	59	63	64	63	58	65	64	77	52	71	103	64	59	58	69	76	76	86	75	98	91	78	68	84	86	
4 Consobrinus	29,8	9,2	52	28	36		84	43	70	77	84	87	91	88	84	73	86	78	85	65	81	65	66	80	77	92	66	79	101	83	77	69	79	80	86	99	80	107	97	82	80	92	88	
5 Cryptarum	27,5	12,2	52	22	30	28		90	74	69	80	83	79	53	4	33	55	36	71	82	69	76	70	74	69	78	69	76	99	63	72	73	51	81	83	97	91	109	110	90	78	94	100	
6 Hortorum	27,4	9,0	52	24	33	9	25		69	77	87	84	94	90	88	80	86	89	84	78	83	74	66	82	79	94	68	87	104	86	74	69	80	83	94	109	90	107	104	93	83	87	101	
7 Humilis	26,3	9,5	49	20	29	26	23	23		58	75	78	82	77	72	76	85	77	72	66	76	32	26	61	71	78	30	75	95	64	58	37	80	73	85	84	76	99	89	75	69	80	94	
8 Hyperboreus	25,9	9,2	45	6	24	27	26	23	22		65	74	76	70	69	61	71	69	70	69	64	65	58	48	66	75	63	72	103	66	61	54	66	70	70	76	72	87	80	68	59	69	74	
9 Hypnorum	25,3	9,6	47	21	23	25	25	24	22	21		59	74	79	77	78	85	87	70	75	56	80	75	76	52	62	82	70	111	75	73	73	79	81	83	91	82	93	91	81	68	83	92	
10 Jonellus	30,5	8,8	52	26	28	27	30	27	30	27	17		83	82	82	79	81	87	70	80	59	77	72	77	51	59	81	77	96	70	69	74	80	77	85	101	87	109	99	90	75	96	106	
11 Lapidarius	33,8	9,8	54	30	35	31	32	28	32	29	23	29		82	79	76	86	85	72	86	70	88	80	76	71	84	78	54	110	70	76	82	81	68	90	93	85	113	104	91	79	94	96	
12 sp. A	28,0	10,6	53	20	33	28	18	25	22	24	21	30	31		53	41	56	56	71	82	72	80	80	78	66	80	78	75	102	54	73	79	48	81	84	98	92	105	100	85	75	93	98	
13 Lucorum	25,5	12,1	50	20	28	26	1	23	21	24	23	28	30	16		33	54	36	70	82	67	74	68	74	67	80	67	75	99	62	71	71	50	82	84	97	91	109	109	91	77	93	99	
14 Lucorum GB	25,8	12,0	48	20	28	27	8	24	21	24	21	28	30	14	6		44	16	68	68	70	74	73	69	62	82	71	69	100	57	66	72	37	82	80	87	79	99	96	83	76	90	90	
15 Maderensis	25,8	12,1	50	19	25	28	11	25	24	21	20	29	27	17	9	9		53	75	80	75	82	81	72	73	85	81	81	100	68	68	73	6	89	86	101	86	99	100	92	81	90	101	
16 Magnus	26,5	12,1	51	21	29	28	7	25	21	25	22	29	31	15	5	3	10		77	75	79	78	75	77	68	88	73	74	101	63	75	76	47	92	84	92	84	105	102	87	80	94	96	
17 Mendax	32,6	8,7	43	30	22	28	32	29	34	27	24	26	27	35	30	32	28	33		75	71	80	71	74	70	74	76	64	95	64	65	77	72	73	87	99	82	106	102	80	70	94	86	
18 Mesomelas	23,2	9,9	50	17	26	21	19	18	13	17	15	25	25	18	17	19	18	20	28		72	58	61	77	72	84	60	70	104	78	69	63	73	83	73	81	71	94	91	81	72	85	85	
19 Monticola	26,5	9,1	47	20	26	23	24	23	26	22	11	16	28	22	22	20	23	21	22	19		74	72	74	44	56	73	64	108	79	71	68	68	71	78	91	85	103	93	85	76	90	91	
20 Muscorum	24,6	9,6	48	18	25	22	23	19	10	17	20	28	28	22	21	21	22	22	32	11	24		33	69	74	80	29	75	97	70	61	33	76	76	77	83	69	95	85	74	71	78	87	
21 Pascuorum	22,0	9,2	48	15	28	20	18	15	10	16	19	23	25	18	16	16	19	17	29	11	21	8		62	69	76	29	68	97	71	59	32	76	69	78	80	71	98	85	77	65	82	90	
22 Polaris	28,0	10,0	50	11	28	31	23	27	27	10	23	30	29	23	21	21	18	22	29	23	23	23	21		79	81	67	74	107	71	61	60	69	75	77	84	77	105	96	81	74	87	93	
23 Pratorum	28,2	11,0	52	20	27	25	26	26	26	21	10	19	25	24	24	26	21	25	26	17	16	24	22	23		44	74	67	97	64	76	73	68	70	77	95	82	107	95	83	66	88	95	
24 Pyrenaeus	24,6	10,2	46	19	20	21	25	20	20	19	10	15	25	23	23	21	22	22	22	13	13	18	18	23	10		81	80	97	77	74	77	77	71	87	96	80	106	85	93	77	93	100	
25 Ruderarius	23,6	10,2	46	17	28	21	20	18	9	19	19	25	27	19	18	18	21	19	29	8	21	9	7	24	21	15		73	93	75	61	23	75	81	82	87	76	107	95	83	72	88	91	
26 Sicheli	27,5	8,8	45	24	24	19	29	18	28	19	19	25	24	24	27	27	22	28	22	20	22	25	23	22	21	17	23		99	70	66	72	76	69	82	90	84	97	93	77	69	87	83	
27 Soroeensis	36,1	6,8	53	35	38	34	32	30	35	34	32	37	38	35	30	29	29	30	41	35	31	31	28	32	36	32	32	34		94	95	96	97	97	99	105	94	117	110	101	102	106	111	
28 Sporadicus	29,6	8,3	52	24	34	33	21	31	28	25	26	33	32	24	19	19	18	20	33	24	25	26	23	23	29	26	25	28	34		68	71	64	72	82	86	78	100	88	73	72	80	91	
29 Subterraneus	22,6	8,8	43	15	25	19	19	16	13	17	21	25	28	19	17	18	17	19	26	14	23	13	9	18	23	17	10	21	30	22		56	64	69	82	95	82	99	93	81	66	85	94	
30 Sylvarum	24,0	10,2	47	16	24	24	21	21	10	18	20	24	27	20	19	19	18	20	29	11	24	8	8	23	22	16	7	24	33	26	9		70	75	79	82	77	102	89	82	70	87	86	
31 Terrestris	24,4	10,7	45	19	23	25	11	25	24	21	19	26	27	18	9	9	3	10	26	19	20	22	19	18	20	20	21	22	26	15	17	19		84	82	93	78	93	90	86	76	85	93	
32 Wurfleini	24,7	8,1	41	24	21	23	23	20	23	19	17	24	22	25	21	23	18	24	19	17	22	19	17	19	20	17	18	11	28	28	16	19	18		82	100	83	106	93	85	74	86	94	
33 Barbutellus	39,9	8,7	57	42	45	46	46	41	42	41	40	42	51	45	44	45	46	45	47	41	39	42	36	44	48	42	43	42	47	43	38	44	43	40		69	54	80	73	62	55	72	62	
34 Bohemicus	37,8	8,1	52	42	42	43	44	40	39	39	40	45	50	45	42	44	43	45	38	38	39	39	34	42	46	42	38	39	44	40	34	38	38	36	26		70	71	73	71	68	74	63	
35 Campestris	34,8	7,2	57	36	39	38	40	34	29	35	33	38	44	39	38	38	41	38	45	27	37	29	28	40	39	31	30	38	47	38	31	31	38	35	28	30		72	64	55	56	64	61	
36 Flavidus	38,5	10,4	60	41	46	45	47	43	37	40	42	45	51	44	45	45	46	45	45	38	42	37	33	44	46	43	38	43	47	40	36	39	43	39	22	22	24		48	50	69	54	73	
37 Norvegicus	34,1	9,4	54	36	42	38	40	36	34	35	37	40	49	39	38	40	41	40	43	33	36	34	30	39	39	37	35	37	43	35	31	36	38	34	21	17	22	11		48	63	50	71	
38 Quadricolor	34,2	9,1	53	37	39	39	41	35	32	35	37	40	46	38	39	40	41	40	44	33	39	32	28	41	43	37	33	37	44	34	28	34	38	33	21	22	22	15	13		52	35	61	
39 Rupestris	40,7	7,8	59	41	43	46	48	44	39	40	43	46	50	47	46	46	45	47	52	36	47	37	35	45	45	43	36	44	49	45	37	35	41	38	32	29	33	28	26	28		59	60	
40 Sylvestris	35,8	10,2	56	39	44	40	43	36	35	37	39	42	50	43	41	42	41	42	48	34	39	35	31	43	45	39	36	40	44	38	31	35	40	36	18	22	22	15	13	10	24		69	
41 Vestalis	40,3	7,9	57	47	48	43	46	40	42	43	40	45	51	43	44	42	47	43	50	39	38	40	37	44	48	42	41	43	46	44	40	42	41	41	29	23	29	29	22	28	31	24		
Total	29,8	11,3																																										

Table 6. Frequencies of the four bases.

	CO-I G	*EF-1α* G	*CO-I* A	*EF-1α* A	*CO-I* C	*EF-1α* C	*CO-I* T	*EF-1α* T	*CO-I* A+T	*EF-1α* A+T
1 *Apis mellifera*	0,12	0,30	0,33	0,26	0,13	0,24	0,42	0,21	0,75	0,47
2 *B. balteatus*	0,13	0,29	0,34	0,26	0,11	0,24	0,43	0,21	0,77	0,47
3 *B. confusus*	0,11	0,30	0,33	0,25	0,12	0,26	0,44	0,20	0,77	0,45
4 *B. consobrinus*	0,12	0,29	0,33	0,25	0,13	0,25	0,43	0,21	0,76	0,46
5 *B. cryptarum*	0,12		0,33		0,13		0,42		0,75	
6 *B. hortorum*	0,12	0,29	0,33	0,26	0,13	0,25	0,42	0,20	0,75	0,46
7 *B. humilis*	0,12		0,33		0,11		0,44		0,77	
8 *B. hyperboreus*	0,12	0,29	0,34	0,26	0,11	0,25	0,42	0,21	0,76	0,47
9 *B. hypnorum*	0,11	0,29	0,33	0,26	0,13	0,25	0,42	0,21	0,75	0,47
10 *B. jonellus*	0,12	0,29	0,35	0,26	0,13	0,25	0,41	0,20	0,76	0,46
11 *B. lapidarius*	0,11	0,29	0,33	0,26	0,14	0,25	0,42	0,20	0,75	0,46
12 *B.* sp. A	0,11		0,35		0,13		0,41		0,76	
13 *B. lucorum*	0,12	0,29	0,33	0,26	0,12	0,25	0,42	0,20	0,75	0,46
14 *B. lucorum* GB	0,11		0,34		0,12		0,43		0,77	
15 *B. maderensis*	0,11		0,34		0,13		0,41		0,75	
16 *B. magnus*	0,12		0,34		0,12		0,42		0,76	
17 *B. mendax*	0,12	0,30	0,31	0,25	0,13	0,26	0,43	0,19	0,74	0,44
18 *B. mesomelas*	0,11	0,29	0,33	0,26	0,11	0,25	0,44	0,20	0,77	0,46
19 *B. monticola*	0,11	0,29	0,34	0,26	0,12	0,25	0,42	0,20	0,76	0,46
20 *B. muscorum*	0,12		0,33		0,11		0,44		0,77	
21 *B. pascuorum*	0,12	0,29	0,33	0,26	0,11	0,25	0,44	0,21	0,77	0,47
22 *B. polaris*	0,12		0,33		0,11		0,43		0,76	
23 *B. pratorum*	0,11	0,29	0,34	0,26	0,12	0,25	0,42	0,20	0,76	0,46
24 *B. pyrenaeus*	0,11	0,29	0,33	0,26	0,13	0,25	0,42	0,21	0,75	0,47
25 *B. ruderarius*	0,12	0,29	0,33	0,26	0,11	0,25	0,44	0,20	0,77	0,46
26 *B. sicheli*	0,11		0,33		0,13		0,43		0,76	
27 *B. soroeensis*	0,12	0,29	0,33	0,25	0,15	0,25	0,39	0,20	0,72	0,45
28 *B. sporadicus*	0,11	0,29	0,34	0,26	0,12	0,25	0,42	0,20	0,76	0,46
29 *B. subterraneus*	0,12	0,30	0,33	0,25	0,11	0,25	0,44	0,20	0,77	0,45
30 *B. sylvarum*	0,12		0,33		0,11		0,43		0,76	
31 *B. terrestris*	0,11	0,29	0,34	0,26	0,13	0,25	0,40	0,20	0,74	0,46
32 *B. wurfleini*	0,12	0,29	0,32	0,26	0,13	0,25	0,43	0,21	0,75	0,47
33 *P. barbutellus*	0,11	0,29	0,35	0,26	0,13	0,25	0,42	0,21	0,77	0,47
34 *P. bohemicus*	0,11	0,29	0,34	0,26	0,15	0,25	0,40	0,20	0,74	0,46
35 *P. campestris*	0,11	0,29	0,33	0,26	0,11	0,25	0,44	0,20	0,77	0,46
36 *P. flavidus*	0,11	0,29	0,34	0,26	0,15	0,25	0,40	0,20	0,74	0,46
37 *P. norvegicus*	0,11		0,35		0,14		0,40		0,75	
38 *P. quadricolor*	0,11		0,34		0,13		0,42		0,76	
39 *P. rupestris*	0,11	0,29	0,34	0,26	0,12	0,25	0,42	0,20	0,76	0,46
40 *P. sylvestris*	0,11		0,34		0,13		0,41		0,75	
41 *P. vestalis*	0,11		0,34		0,14		0,41		0,75	
Mean	0,11	0,29	0,33	0,26	0,12	0,25	0,42	0,20	0,76	0,46
SD	0,01	0,004	0,01	0,004	0,01	0,004	0,01	0,005		

position. Fifteen polymorphic sites were observed in *B. terrestris*. Most of the variations (12) are transitions and all are third position substitutions. The English genotype differs in 13 sites from the two Danish genotypes. Sixteen polymorphic sites were observed in *B. hyperboreus*, of which 12 were transitions (10 3rd positions, one 1st and one 2nd position) and four were transversions, of which 3 are in 3rd positions and one in 1st position. The Norwegian specimen seems closer to one of the Greenlandic genotypes than the two Greenlandic genotypes are to each other. Two polymorphic sites were observed in *B. lucorum* from Central and North Europe. The English specimens identified as *B. lucorum* differ from the European material in adding 38 polymorphic sites extra; 6 are substitutions in 1st positions of the codons. The English specimens are included with the genotype from Central Europe in the phylogenetic analyses. The observed variation in the polymorphic sites is remarkable because the transition/transversion ratio of the total material of poly-

Table 7. Codon usage in percent of total in *mtCO-I* (1056 bp in 40 species) and in *EF-1α* (1152 bp in 26 species).

Codon	Amino acid	CO-I	EF-1α	Codon	Amino acid	CO-I	EF-1α	Codon	Amino acid	CO-I	EF-1α	Codon	Amino acid	CO-I	EF-1α
TTT	Phe	8,5	0,2	TCT	Ser	2,6	0,3	TAT	Tyr	3,5	0,3	TGT	Cys	0,3	0,2
TTC	Phe	0,7	3,7	TCC	Ser	0,3	0,8	TAC	Tyr	0,3	2,3	TGC	Cys	0,0	1,1
TTA	Leu	9,7	0,5	TCA	Ser	4,2	0,0	TAA	Ter	0,0	0,0	TGA	Trp	2,5	0,0
TTG	Leu	0,1	2,1	TCG	Ser	0,0	2,1	TAG	Ter	0,0	0,0	TGG	Trp	0,0	1,3
CTT	Leu	1,3	0,8	CCT	Pro	2,3	0,3	CAT	His	3,1	0,8	CGT	Arg	0,0	2,2
CTC	Leu	0,0	0,5	CCC	Pro	0,1	0,8	CAC	His	0,3	1,3	CGC	Arg	0,0	1,0
CTA	Leu	0,8	0,0	CCA	Pro	2,4	0,7	CAA	Gln	1,7	0,6	CGA	Arg	0,8	0,3
CTG	Leu	0,0	2,6	CCG	Pro	0,0	4,4	CAG	Gln	0,0	2,0	CGG	Arg	0,0	0,0
ATT	Ile	11,5	1,0	ACT	Thr	2,3	1,2	AAT	Asn	4,8	0,9	AGT	Ser	0,2	0,3
ATC	Ile	0,5	5,5	ACC	Thr	0,1	3,0	AAC	Asn	0,3	2,7	AGC	Ser	0,0	0,8
ATA	Met/Ile	6,9	0,5	ACA	Thr	3,6	0,4	AAA	Lys	2,0	4,6	AGA	Ser/Arg	1,5	0,5
ATG	Met	0,1	2,3	ACG	Thr	0,0	1,9	AAG	Lys	0,0	4,3	AGG	Ter/Arg	0,0	0,0
GTT	Val	2,7	0,0	GCT	Ala	1,4	4,0	GAT	Asp	2,6	2,1	GGT	Gly	2,2	4,8
GTC	Val	0,0	1,4	GCC	Ala	0,1	2,2	GAC	Asp	0,2	2,8	GGC	Gly	0,1	1,7
GTA	Val	2,3	1,6	GCA	Ala	2,0	0,8	GAA	Glu	0,9	3,2	GGA	Gly	5,8	1,0
GTG	Val	0,0	4,0	GCG	Ala	0,0	2,0	GAG	Glu	0,0	4,1	GGG	Gly	0,1	1,0

norphisms (excluding the English *B. lucorum*) is 53:33. Only in *B. mendax* and *B. sylvarum* are ransversions more numerous than transitions. This tendency of a generally high transition/transversion ratio in the intraspecific variation contrasts with a low ratio when all taxa are compared (see below).

No deletions or insertions of bases were observed in the aligned sequences of the studied *mtCO-I* region in *Apis mellifera* and the forty umblebee taxa.

Among all the taxa studied in the phylogenetic nalyses (including the honeybee), 443 of the sites 42%) were variable (147 in 1st+2nd positions of ie codon; 296 in 3rd position). The bumblebees ncluding the cuckoo bumblebees) are identical to ich other and different from the honey bee in ily 15 sites (1.4%). Fig. 1 illustrates the distribuon of variable sites and phylogenetically informive sites on codon positions (including *Apis:* 75 1st, 17 in 2nd, 234 in 3rd; excluding *Apis:* 70 in t, 16 in 2nd, 232 in 3rd) among the studied taxa. both comparisons, including or excluding *Apis*, e majority of the phylogenetically informative es are in third positions.

Because the mitochondrial *CO-I* gene is known a conservative protein-coding gene evolving der functional constraints, most of the substituns are expected to be in third positions - primaı silent substitutions. Tab. 5 shows a matrix of observed substitutions by codon positions. The culated mean ± SD for first + second position substitutions for all the comparisons is 29.8 ±11.3 and for third position 78.5 ± 15.7. Generally, substitutions in third positions are two to three times the number of first + second position substitutions. The undescribed Norwegian *Bombus* sp. A has the shortest distance in number of substitutions to species within the subgenus *Bombus* (*B. cryptarum*, *B. lucorum*, *B. maderensis*, *B. magnus*, *B. sporadicus*, *B. terrestris*); there is a distance of 14+41 substitutions to the nearest taxon (*B. lucorum* GB). Only a small distance (1+4) was observed between *B. cryptarum* and *B. lucorum*, but *B. cryptarum* is here accepted as a separate taxon because the haplotype was observed both in Austria and in Denmark and in both countries is sympatric with *B. lucorum*. Tab. 6 summarizes the frequencies of the four nucleotides distributed over the 41 taxa. There are only small differences among the taxa in *mtCO-I*: G ranges from 0.11-0.13, C from 0.11 to 0.15, A from 0.33-0.35, and T from 0.39-0.44, indicating a strong A-T bias. This bias also is distinct in the usage of codons (see Tab. 7). In all cases, codons ending in an A or T are definitely more common than G and C. The bias is so strong that the observed variation can be regarded as a variation in 'two dimensions' instead of four. The extremely high A-T bias seems to be characteristic in the Apocrita (Hymenoptera) (Dowton & Austin 1995).

Fig. 2 compares frequencies of the transitions and transversions in 3rd positions in relation to the frequencies of substitutions in 1st plus 2nd posi-

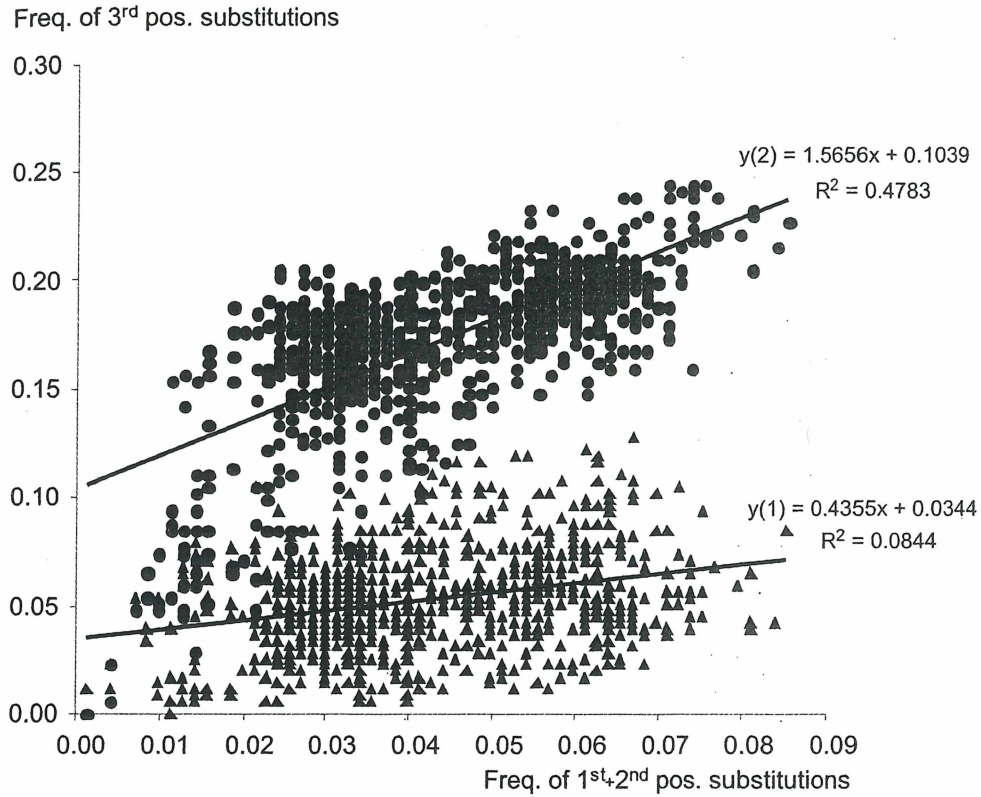

Figure 2. Frequencies of 3rd position substitutions (transitions ▲y(1), transversions ●y(2)) and the frequencies of all 1st plus 2nd position substitutions (x) for all comparisons in *mtCO-I*. Estimated regression lines for y(1) and y(2) are shown. Coefficient of determination, R^2.

tions, including estimated regression lines in order to analyse eventual saturation. The curve fitting the frequencies of the transitions indicates for higher rate values in 1st plus 2nd positions saturation in tending towards a horizontal line. On the other hand, the transversions show a weak tendency to saturation only for the highest rates. The observed saturation index was also tested against half of full substitution saturation (Xia 2000). The null hypothesis that there is correspondence between half of the full saturation and the observed saturation index is rejected ($P<0.001$ for $t= 30.1$ df 76).

Elongation Factor 1α

The studied region in *EF-1α*, was easily aligned to *EF-1α* in *Apis mellifera* (F1) in Danforth & Ji (1998); it consists of two parts of two exons and an intervening intron. No intraspecific variation in specimens from different localities of the studied 26 species was observed. Only the exons (703 + 450 bp) were used in the study. The lengths of the introns vary from species to species - from 181 nucleotides in *B. lucorum* to 403 in *B. ruderarius*. In *Apis*, the corresponding length is 219 nucleotides. The introns were omitted from the analyses because of the considerable length differences and because only minor parts of the introns could be aligned to *Apis*. In the aligned regions of the two exons, no deletions or insertions of bases relative to the *Apis* sequence were observed.

Among all the taxa studied in the phylogenetic

Table 8. Matrix of observed substitutions for first + second positions (below diagonal) and third positions (above) in the *EF-1 alpha* codons.

	Means		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	Total
		SD	113.2	24.0	70.0	30.9	29.9	23.0	25.9	26.0	28.9	25.9	42.2	25.2	28.3	24.9	23.6	24.0	26.3	26.3	27.7	34.7	27.1	27.2	25.8	23.8	22.9	25.2	25.7	31.8
			5.5	20.9	13.4	18.4	20.3	21.1	21.6	20.8	20.3	20.9	15.6	21.0	20.6	21.2	21.4	22.1	20.6	18.1	20.9	18.2	20.5	20.8	21.5	21.6	21.1	20.6	21.5	19.7
1 A. mellifera	7.2	1.4		110	134	108	116	111	113	114	114	109	115	115	116	113	111	114	113	102	110	116	107	116	116	114	110	110	116	
2 Balteatus	2.4	1.8	7		68	25	24	3	15	17	18	14	37	18	19	18	13	13	18	21	17	30	16	18	20	18	16	20	19	
3 Confusus	7.1	1.4	10	7		69	68	65	73	65	72	69	60	66	65	69	69	71	67	65	72	64	71	68	66	66	65	66	66	
4 Consobrinus	4.2	1.9	9	4	9		12	24	28	25	29	27	38	22	30	25	24	26	25	24	32	33	29	29	25	23	23	23	26	
5 Hortorum	3.2	1.9	8	3	8	1		23	27	26	26	27	42	19	29	22	25	25	22	21	31	34	29	26	22	20	20	20	21	
6 Hyperboreus	3.4	1.8	8	3	8	5	4		14	14	19	13	36	17	18	17	12	12	17	20	16	29	15	15	19	17	15	19	18	
7 Hypnorum	1.5	1.8	6	1	6	3	2	2		13	23	18	40	20	14	20	7	7	23	23	19	29	19	18	23	21	19	23	24	
8 Jonellus	2.3	2.0	7	2	7	4	3	3	1		24	21	38	18	15	22	8	10	24	22	22	25	21	18	24	22	20	24	23	
9 Lapidarius	2.4	1.8	7	2	7	4	3	3	1	2		22	37	21	27	22	21	21	20	25	25	34	24	22	22	20	20	22	21	
10 Lucorum	2.3	1.9	7	2	7	4	3	3	1	2	2		41	22	23	22	17	18	22	21	5	36	2	22	22	20	18	22	21	
11 Mendax	7.2	1.6	12	7	12	9	8	8	6	7	7	7		37	38	37	36	38	39	38	44	37	43	39	39	35	37	39	38	
12 Mesomelas	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6		25	15	19	19	17	19	24	27	24	17	16	14	14	16	15	
13 Monticola	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6	0		25	11	11	23	24	22	29	23	21	27	25	23	25	28	
14 Pascuorum	2.3	1.9	7	2	7	4	3	3	1	2	2	2	7	1	1		19	17	12	17	23	26	24	20	14	10	10	14	15	
15 Pratorum	2.3	2.0	7	2	7	4	3	3	1	0	2	2	7	1	1	2		4	21	19	18	25	17	17	21	19	17	21	22	
16 Pyrenaeus	2.3	2.0	7	2	7	4	3	3	1	0	2	2	7	1	1	2	0		19	20	19	25	18	17	21	19	17	21	22	
17 Ruderarius	2.3	1.9	7	2	7	4	3	3	1	2	2	2	7	1	1	0	2	2		19	23	32	24	22	18	16	14	18	17	
18 Soroeensis	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6	0	0	1	1	1	1		22	29	21	23	19	17	17	17	20	
19 Sporadicus	2.3	1.7	7	2	5	4	3	3	1	2	2	2	7	1	1	2	2	2	2	1		33	5	21	25	23	21	25	24	
20 Subterraneus	2.4	1.8	7	2	7	4	3	3	1	2	2	2	7	1	1	2	2	2	2	1	2		36	30	30	26	28	30	29	
21 Terrestris	2.3	1.9	7	2	7	4	3	3	1	2	2	0	7	1	1	2	2	2	2	1	2	2		24	24	22	20	24	23	
22 Wurfleini	2.4	1.8	7	2	7	4	3	3	1	2	2	2	7	1	1	2	2	2	2	1	2	2	2		22	20	20	22	21	
23 Barbutellus	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6	0	0	1	1	1	1	0	1	1	1	1		8	8	10	11	
24 Bohemicus	2.4	1.8	7	2	7	4	3	3	1	2	2	2	7	1	1	2	2	2	2	1	2	2	2	2	1		6	8	9	
25 Campestris	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6	0	0	1	1	1	1	0	1	1	1	1	0	1		8	9	
26 Flavidus	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6	0	0	1	1	1	1	0	1	1	1	1	0	1	0		9	
27 Rupestris	1.5	1.8	6	1	6	3	2	2	0	1	1	1	6	0	0	1	1	1	1	0	1	1	1	1	0	1	0	0		
Total	2.8	1.8																												

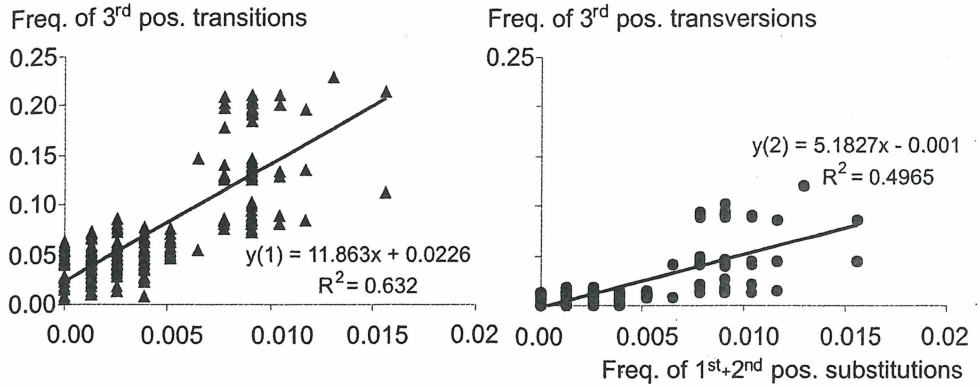

Figure 3. Frequencies of third position substitutions (transitions ▲y(1), transversions ●y(2)) and the frequencies of all first plus second position substitutions (x) for all com-parisons in EF-I-a. Estimated regression lines for y(1) and y(2) are shown. Coefficient of determination, R^2.

analyses (including the honeybee), 232 of the sites (20%) in the exons were variable (30 in 1st+2nd positions of the codon; 202 in 3rd position). The bumblebees (including the cuckoo bumblebees) are identical to each other, but differ from the honeybee, in 58 sites (5.0%). The distribution of variable sites and phylogenetically informative sites on codon positions (including *Apis:* 7 in 1st; 0 in 2nd; 90 in 3rd; excluding *Apis:* 6 in 1st; 0 in 2nd; 72 in 3rd) among the studied taxa are shown in Fig. 1. The vast majority of phylogenetically informative sites are in third positions in both comparisons.

EF-1α is known as a conservative protein-coding gene and has been used in phylogenetic analyses at higher levels of the hierarchical system. Most of the substitutions are expected to be in third positions and thus primarily silent substitutions. Tab. 8 shows a matrix of the observed substitutions by codon positions. The calculated mean ± SD for 1st + 2nd position substitutions for all the comparisons is 2.8 ±1.8 and for 3rd positions 31.8 ±19.7. This means the number of substitutions in 3rd positions are more than ten times that of 1st + 2nd position substitutions. Tab. 6 summarize the frequencies of the four nucleotides distributed over the 26 studied taxa. Only small differences among the taxa were observed in *EF-1α*: G ranges from 0.29-0.30, C from 0.24 to 0.26, A from 0.25-0.26, and T from 0.19-0.21, indicating a homogeneous distribution of bases. In contrast to the conditions in *mtCO-I* codons, codons ending in a C or G are, with few exceptions, more common than those ending with A and T (see Tab. 7). Fig. 3 compares the frequencies of the transitions and the transversions in 3rd positions to the frequencies of substitutions in 1st and 2nd positions. These comparisons are used to analyse eventual saturation. There is no tendency towards a horizontal line in the curves fitting the relationships, meaning no saturation was observed. The observed saturation index was also tested against half of full substitution saturation (Xia 2000). The null hypothesis that there is correspondence between half of the full saturation and the observed saturation index is rejected ($P<0.001$ for $t= 55.4$ df 33).

The two studied DNA regions have evolved under different constraints. The mitochondrial region in *CO-I* has a pronounced A-T bias as opposed to the condition in *EF-1α*, which has a more homogenous distribution of nucleotides. The substitution rates based on all comparisons have means for 3rd positions that are two to three times the rates for 1st plus 2nd positions in CO-I, whereas the corresponding means in *EF-1α* are six to twenty times greater.

The *CO-I* segment was used to derive the phylogeny in Fig. 4 using maximum parsimony (MP). All positions are equally weighted. Both the heuristic search in DNAPARS (Felsenstein 2000), as well as the corresponding algorithm in PAUP (Swofford 2000), were used with randomised input order of taxa. The multistates of some of the taxa were run both with the multistates treated as

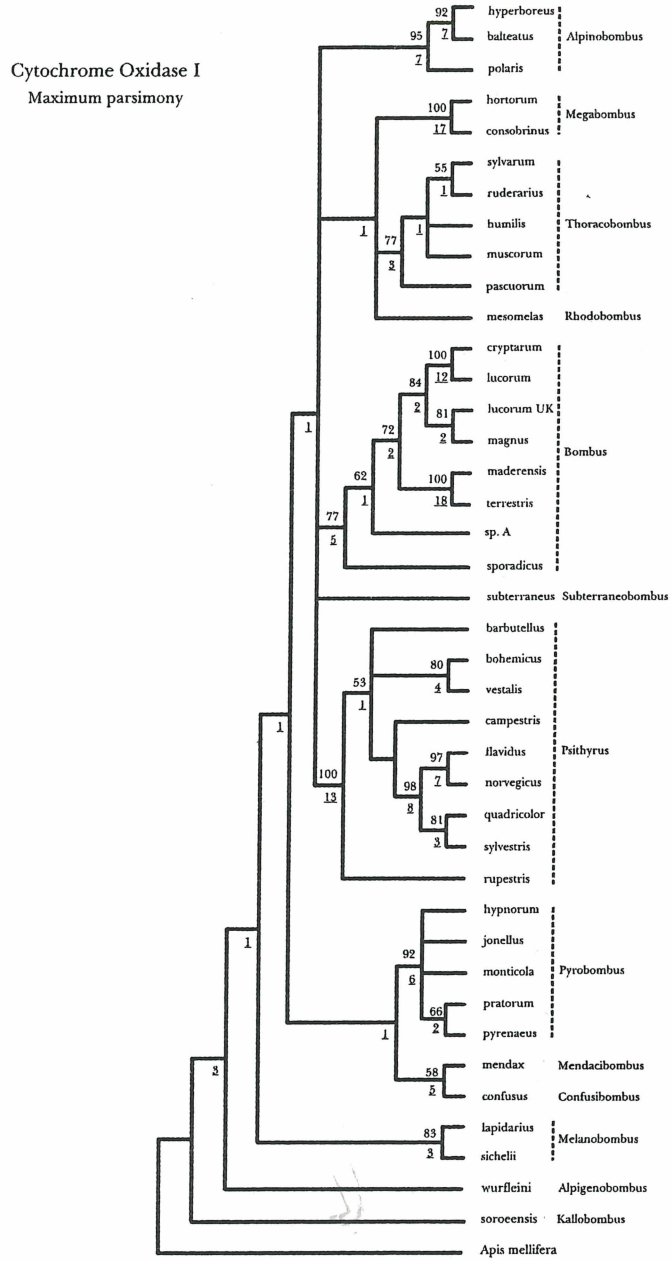

Figure 4. Strict consensus tree based upon the 39 shortest trees, all with the length of 1763 evolutionary steps in maximum parsimony analyses of the studied region of *mtCO-I* in 40 bumblebee taxa. Bootstrap values in percentage based on 2 analyses of 1000 replicates are shown above the branches and 'decay indices' (Bremer 1994) under the branches. The commonly accepted subgenera are indicated on the tree. *Apis mellifera* is used as outgroup.

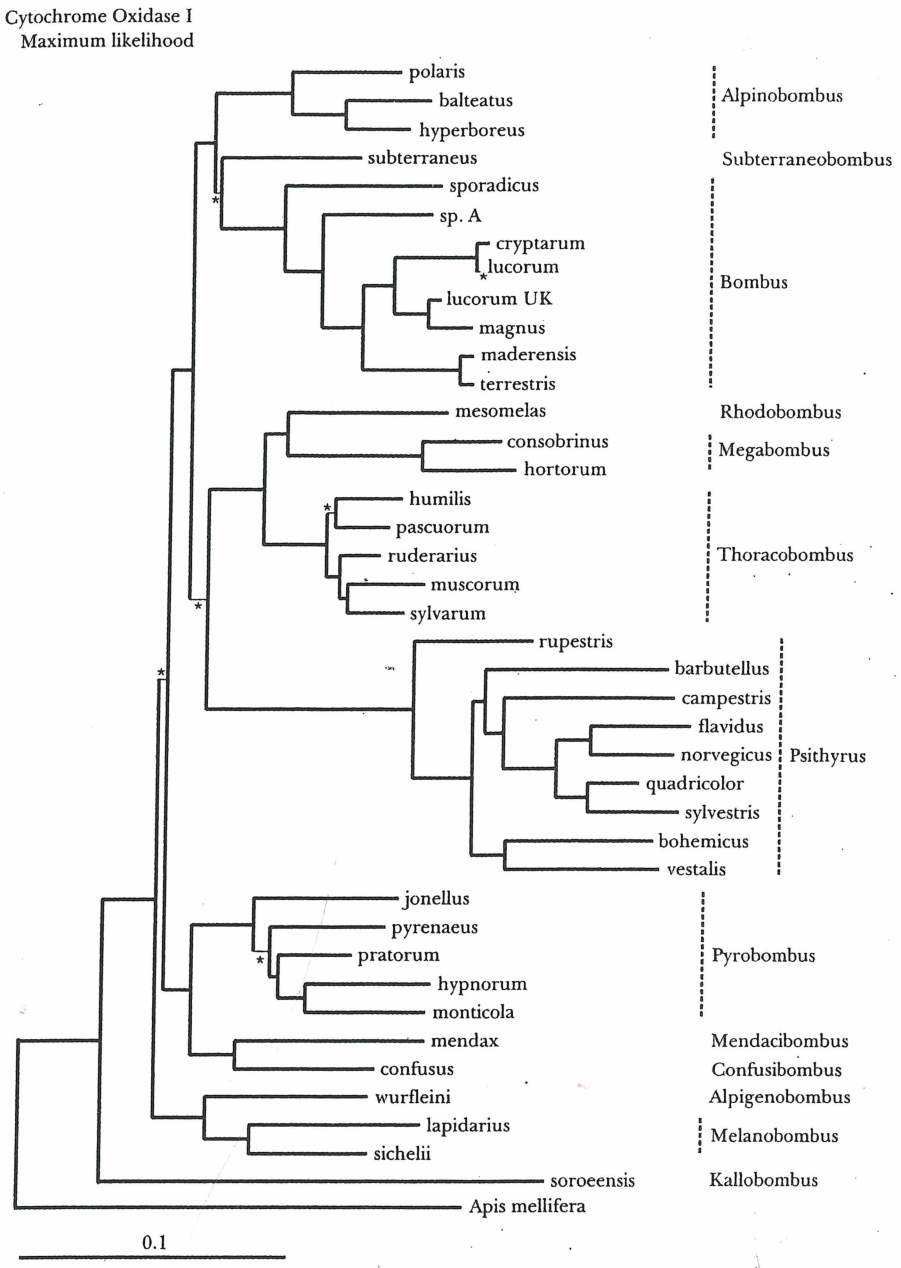

Figure 5. Phylogeny based on maximum likelihood analyses of the studied region of *mtCO-I* in 40 bumblebee taxa. ln-likelihood value was -9562.18354 based on Ti/Tv=0.87, the α parameter = 0.22 and 8 rate categories. A * indicates branch lengths which do not differ significantly from zero. The commonly accepted subgenera are indicated on the tree. *Apis mellifera* is used as outgroup.

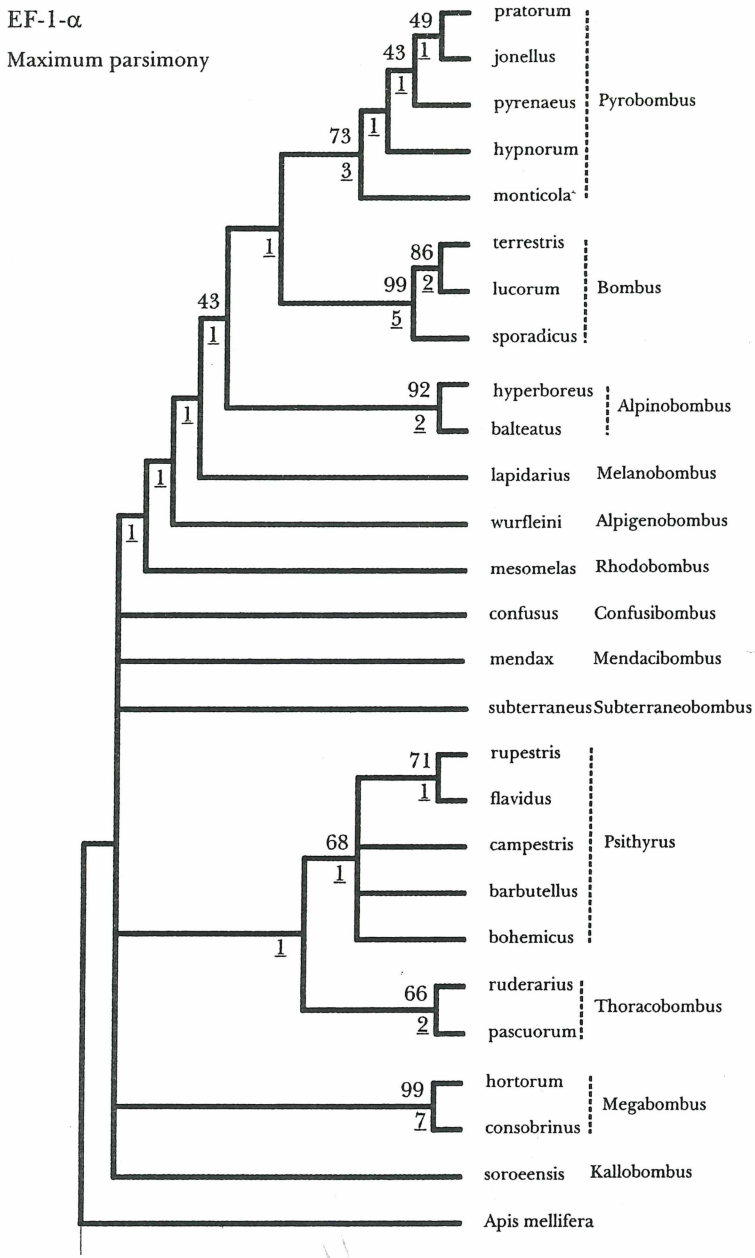

Figure 6. Strict consensus tree based upon the 4 shortest trees, all with the length of 384 evolutionary steps in maximum parsimony analyses of the studied region in the nuclear *EF-1α* sequences from 26 species of bumblebees. Bootstrap values in percentage based upon an analysis of 1000 replicates are shown above the branches and 'decay indices' (Bremer 1994) under the branches. The commonly accepted subgenera are indicated on the tree. *Apis mellifera* is used as outgroup.

uncertainties and as polymorphisms (PAUP). The 39 shortest trees were based on 1763 evolutionary steps. Multistate taxa treated with the 'polymorphism option' in PAUP resulted in 36 trees of 1862 steps in length. The consensus tree, which is identical whether it is based on DNAPARS or PAUP (+/- 'polymorphism option'), has values from bootstrap analyses given above the branches and decay indices (Bremer 1994) under the branches. The bootstrap values are percentages based on 2 analyses of 1000 bootstrap replicates. Commonly accepted subgenera (Williams 1994) are indicated by a vertical line. No conflict seems to exist between the terminal groupings and the division of subgenera. Bootstrap values as well as in decay indices strongly support *Alpinobombus*, *Megabombus*, *Psithyrus* and *Pyrobombus* and less strongly support *Thoracobombus*, *Bombus* and *Melanobombus*. Although the clade containing *B. mendax* and *B. confusus (Mendacibombus + Confusibombus)* has a low bootstrap value, there is good support in the decay index for this sister-group relationship.

Maximum-likelihood analyses were conducted with PAUP *. The Hasegawa-Kishino-Yano model of sequence evolution (Hasegawa et al. 1985) using observed nucleotide frequencies, different transition/transversion (Ti/Tv) ratios and different rates of heterogeneities (α in the distribution of variable sites - Γ distribution), were used to optimize the models and to calculate the highest ln-likelihood values. The highest ln-likelihood value was -9562.1835, based on Ti/Tv=0.87, the α parameter = 0.22 and 8 rate categories.

The optimized maximum-likelihood tree is shown in Fig. 5, with the different subgenera indicated. An asterisk (*) indicates branch lengths that do not differ significantly from zero. The different clades correspond very well with the accepted subgenera groupings. The subgenera *Alpinobombus*, *Bombus Megabombus*, *Thoracobombus*, *Psithyrus*, *Pyrobombus* and *Melanobombus* seem to constitute monophyletic entities. The tree closely resembles the MP tree, even in the order of the deepest nodes, but both trees differ in the deepest branchings from the latest published trees based on morphology (Williams 1985, 1991 and 1994). The phylogenetic analyses were also conducted using the *mtCO-I* dataset, excluding all third positions. Most branches on the resulting trees were weakly supported – only 4 terminal clades (*Psithyrus*, *Bombus*, *Megabombus* and *Alpinobombus)* were well supported.

The nuclear *EF-1*α sequences from 26 species of bumblebees and the honey bee were used in the maximum parsimony analyses by PAUP (Swofford 2000) and DNAPARS (Felsenstein 2000). All positions were equally weighted. Both searches in DNAPARS, as well as the corresponding algorithm in PAUP, were used with randomized input order of taxa. The 4 shortest trees were based on 384 evolutionary steps. The consensus tree, which is identical whether based on DNAPARS or PAUP, is shown in Fig. 6, with bootstrap analyses values above the branch and with decay indices (Bremer 1994) under the branch. The bootstrap values are percentages based on 1000 bootstrap replicates. As for the *mtCO-I* analyses, no conflict seems to exist between the terminal groupings and the division of subgenera, although support for some of the clades seems weak. The deeper nodes have so little support that it cannot justify a resolution in dichotomies in the ancestral part of the tree.

As for the *mtCO-I* analyses, the Hasegawa-Kishino-Yano model of sequence evolution (Hasegawa et al. 1985) was used in the Maximum-likelihood analyses. The observed nucleotide frequencies, different transition/tansversion (Ti/Tv) ratios and different rates of heterogeneities (α in the Γ distribution) were used to optimize the models and to calculate the highest ln-likelihood values. The highest ln-likelihood value was -3662.1890 based on Ti/Tv = 3.05, α parameter = 0.188 and 8 rate categories. The resulting phylogeny, including subgenera, is shown in Fig. 7. Some of the branch lengths, indicated on the tree with an asterisk, do not differ significantly from zero. The different clades correspond very well with the accepted grouping of subgenera. The subgenera *Alpinobombus*, *Bombus*, *Megabombus*, *Thoracobombus*, *Psithyrus* and *Pyrobombus* seem to constitute monophyletic entities. As in the other trees, *Alpinobombus* and *Bombus* are grouped together. *B. soroeensis (Kallobombus)* also has a basal position in this analysis. Although several of the internal branches are weakly supported, the tree topography is similar to the other DNA trees and it differs from the published trees based on morphology (Williams 1985, 1991 and 1994).

Discussion

Although both the analyzed DNA segments (the mitochondrial gene *Cytochrome Oxidase I* and the nuclear gene *Elongation factor 1*α*)* are protein-

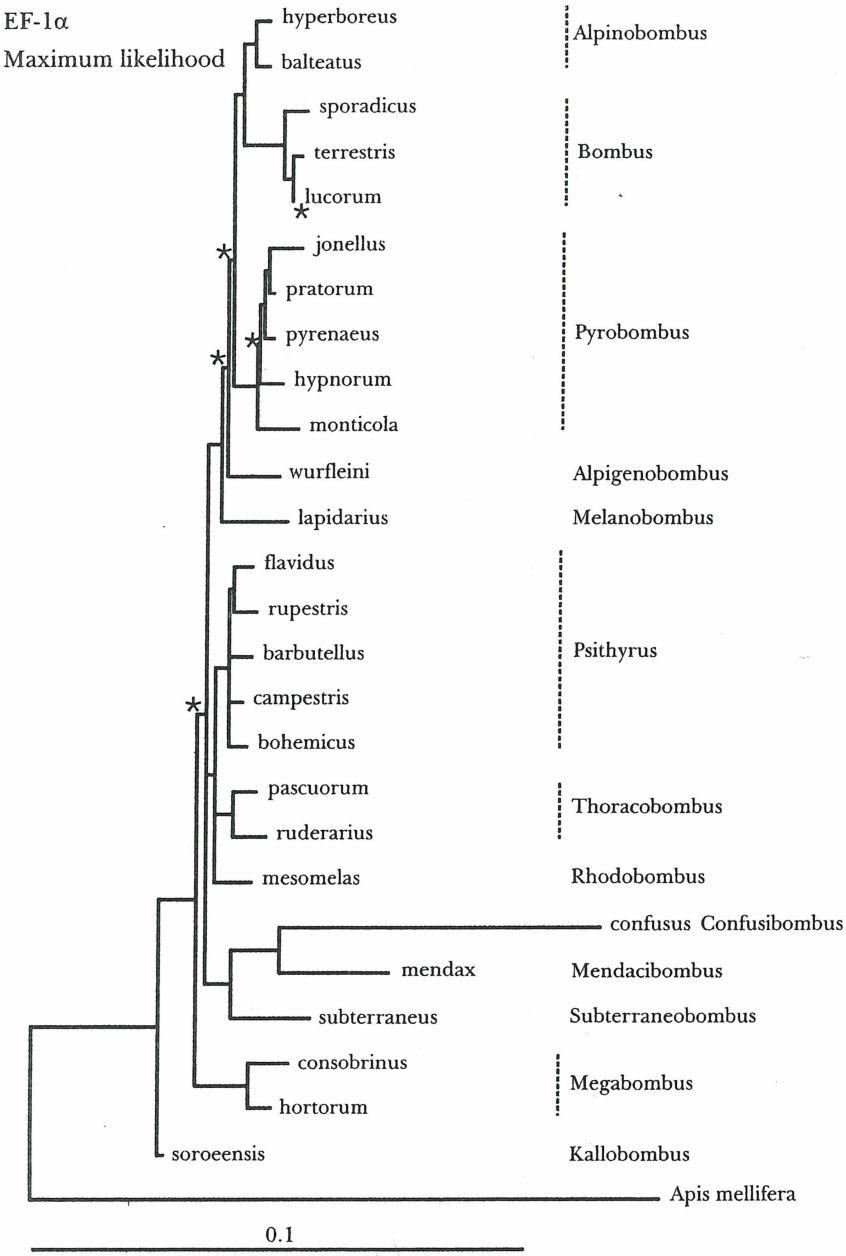

Figure 7. Phylogeny based on maximum likelihood analyses of the studied region of *EF-1α* in 26 species of bumblebees. ln-likelihood value was -3662.18895 based on Ti/Tv = 3.05, the α parameter = 0.188 and 8 rate categories. An * indicates branch lengths which do not differ significantly from zero. The commonly accepted subgenera are indicated on the tree. *Apis mellifera* is used as outgroup.

coding genes, they have differed distinctly in their evolution. In *mtCO-I*, the average observed genetic distance between the studied bumblebees is 0.10 (s.d. 0.02) per site, whereas in *EF -1α*, it is only 0.024 (s.d. 0.014). Differences indicate *EF-1α* is a far more conserved gene than *mtCO-I* and therefore *EF-1α* should be more suitable than *mtCO-I* in the phylogenetic analysis of deeper nodes. The two genes differ considerably in the composition of bases and especially in the observed substitutions. In *EF-1α*, the substitutions are almost equally distributed over the four nucleotides, with substitutions in third positions 10 times the rate of 1st plus 2nd positions. This differs from the condition in the *mtCO-I* segment, because substitutions in third positions are only 3 times the rate of 1st plus 2nd positions and with A↔T transversions are more common than any other substitutions. The A↔T bias is distinctly pronounced in third positions, with an average rate of 0.94 of all 3rd positions transversions versus 0.83 in first plus second positions. This condition results in a biased use of codons. This A↔T bias in mitochondrial DNA is well known in insects, although it is most pronounced in Hymenoptera. The phenomenon has been previously described (Pedersen 1996, Dowton & Austin 1997), but a good explanation of its function is still lacking. The relationships between the distances based on differences in 1st plus 2nd positions of the codons and distances based on transitions and transversions in the 3rd positions indicate that no saturation has occurred. Only the transitions in *mtCO-I* in relation to the largest differences in 1st plus 2nd positions suggest saturation – i.e. a risk that the number of homoplasies decrease the phylogenetic signal of the DNA data.

In summary, the two coding DNA segments differ considerably and should be suitable for phylogenetic analyses at different levels, although the A↔T bias means that the variation in the *mtCO-I* gene is nearly 'two dimensional' instead of the usual 'four dimensional' variation seen in other DNA regions. This condition results in minor variation and perhaps a higher rate of convergences.

The terminal groupings are the same in all the phylogenetic trees, whether based on the *mtCO-I* or *EF-1α* or computed using maximum parsimony or maximum likelihood, and they correspond with the latest analyses based on morphology (Williams 1985, 1991 and 1994). The phylogenetic analyses group the taxa together in the following clusters, all ranking as subgenera: *Alpinobombus*, *Bombus*, *Megabombus*, *Thoracobombus*, *Psithyrus*, *Pyrobombus* and *Melanobombus*. The supports for the groupings are rather strong. In contrast to these groupings, Koulianos (1999) and Pedersen (1996), place *Melanobombus* within *Pyrobombus*. However, the present study is based on a larger segment of *mtCO-I* than was used for the two previous studies and the monophylogeny of *Pyrobombus* and *Melanobombus* seems convincing, although the position of *Melanobombus* in relation to *Pyrobombus* differs from tree to tree. The morphology of the two subgenera indicate close relationships and in his phenetic analyses Ito (1985) places the two subgenera in his '*Pyrobombus*' - group. In the cladistic analyses of Williams (1985 1994), there are obvious distances between the two taxa in spite of only minor differences in the structure of the male genitalia.

The *Alpinobombus* group is well-supported by the present analyses. The three species included occur in the northern part of Europe and are circumpolar. Two of the species, *B. hyperboreus* and *B. polaris*, have been the topic of several studies because *B. hyperboreus* is workerless and usurps the colonies of *B. polaris*. The condition seems to be recent because no morphological adaptations correlated with usurpation have evolved in contrast to the morphological changes observed in *Psithyrus*. A few cases of *B. hyperboreus* with workers are mentioned in the literature, (Löken 1973). Three different haplotypes were seen in the studied material of *B. hyperboreus*, two from Greenland and one from Norway (see Tab. 4). The Norwegian haplotype closely resembles one of the two Greenlandic haplotypes (8 base differences vs. 15).

The *Bombus* group is comprised of 8 taxa based on the *mtCO-I* study. The same sister group relations are observed in the parsimony as well as in the maximum likelihood analyses. *B. cryptarum* and *B. lucorum* differ only in few a substitutions, but *B. cryptarum* is accepted as an independent species because the same haplotype of *B. cryptarum* is found both in Austria and Denmark and the two haplotypes live sympatrically in both countries. The morphology and colour of the specimens agree with the descriptions given in Rasmont (1984). The species *B. terrestris* and *B. maderensis* have distinct colour and morphological differences, but differ only in few substitutions. *B. maderensis* seems to be differentiated

from *B. terrestris* as the consequence of an island population isolated from the main population. This observation corresponds with a study of microsatellite and haplotype (a segment of *mtCytochrome b*) variation in *B. canariensis*, *B. maderensis* and *B. terrestris* from the southern part of Europe (Widmer et al. 1998). Specimens from Scotland identified as typical *B. lucorum* seem to have sister group relations to *B. magnus* from continental Europe. The continental specimens of *B. magnus* have all a typical distinct yellow band continued below the wing base. The same morphotype (*B. magnus)* is known from several localities in England, Scotland and Ireland (Prys-Jones & Corbet,1991). Unfortunately, no English specimens have been available for DNA analyses. The northern Scandinavian species, *B. sporadicus*, is positioned near the root of the *Bombus* group. An undescribed taxon, here stated as *Bombus (Bombus)* sp. A, differs from the other taxa in DNA; its colouring resembles an extremely pale *B. lucorum*. The Scottish specimens of *B. terrestris* differ from the continental *B. terretris* in 13 -15 substitutions (Tab. 4). The Scottish specimens all have a yellow taint in the white terminal band of the abdomen. Although the *Bombus* group of species seems to form a distinct monophyletic group, observed differences within the group indicate taxonomic problems so severe that likely only a closer study of morphology and molecular data from several localities in Europe likely will delimitate the species and solve problems concerning their phylogeny. In the maximum likelihood analyses of *mtCO-I*, the *Bombus* group is linked together with *Alpinobombus*. The analyses of the relationships based on *EF-1α* support this. The maximum parsimony analysis of *mtCO-I* does not contribute any resolution at this level – the result is a polytomi. In all the trees, the cuckoo bumblebees, the *Psithyrus* group, is a well-supported monophyletic group. The sister-group relationships within *Psithyrus* based on *mtCO-I* follow the formerly accepted subgenera divisions within this group: *Fernaldaepsithyrus* (*B. flavidus*, *B. norvegicus*, *B. quadricolor* and *B. sylvestris*), *Ashtonipsithyrus* (*B. bohemicus* and *B. vestalis*) and the rest of the species each placed in its own subgenus *Metapsityrus* (*B. campestris*), *Allopsithyrus* (*B. barbutellus*) and *Psithyrus* (*B. rupestris*). Based on morphology, *Thoracobombus*, *Megabombus*, *Rhodobombus* and *Subterraneobombus* are placed in a group named *Odontobombus*. This group is characterized by, among other features, a spine on the metatarsus of the second pair of legs in females and a long malar area in both sexes (Krüger 1917). Except for *Subterraneobombus*, these relationships are, with greater or lesser support, supported in the shown trees. The close relationship between *Psithyrus* and *Thoracobombus*, postulated in the *mtCO-I* and the *EF-1α* trees based on maximum likelihood, support, to some extent, some former studies that mentioned *Thoracobombus* or the *Odontobombus*-groups as being related to *Psithyrus* (Frison 1927, Richards 1927 and Plowright & Stephen 1973). Ito (1985), in his phenetic analyses stated that patterns in morphology, male genitalia and sterna are characterized by high correlations to *Thoracobombus* and to the other subgenera belonging to the *Odontobombus* group. A cladistic analysis (Ito & Sakagami 1985) supports this view. In the cladistic analyses (Williams 1985, 1994), *Psithyrus* relations to *Thoracobombus* and the other subgenera within *Odontobombus* can only be postulated after removal of a pair of groups from the cladograms. In all the trees, whether based on *mtCO-I or EF-1α*, the *Melanobombus* group is placed as a sister group or close to *Alpigenobombus*, which contradicts Williams (1985 1994), in which *Alpigenobombus* is close to *Pyrobombus*. Ito (1985) also places *Alpigenobombus* and *Melanobombus* close together. All the trees also have *Confusibombus* and *Mendacibombus* placed as sister groups or close together, although the bootstrap values in the *mtCO-I* tree based on parsimony only have a percentage value of 58. On the other hand, the decay index (5) indicates good support for the clade, *Confusibombus* + *Mendacibombus*. In Ito (1985) and especially in Williams (1985, 1991 and 1994), these two groups are placed at the bases of the respective phylogenies. In Williams (1991), *Mendacibombus* is represented by a series of species forming a ladder-like sister group relationship with the rest of the bumblebees, including the cuckoo bumblebees. In Williams (1994), *Confusibombus* has a position close to *Mendacibombus* and the root of the tree. Although several morphological characters segregate both groups, they share features, e.g. males have enlarged, nearly holoptic eyes. Although these two groups are placed near the root in most of the analyses, the *Kallobombus* group, represented by the species *B. soroeensis*, is placed as the most basal node. This is surprising considering the recent morphological analyses. Ito (1985) places

Kallobombus in different positions in his phenograms, although often near *Alpinobombus*. In a single case, *Kallobombus* is placed near *Confusibombus* and the North American *Bombias* group. Williams (1985, 1994) places *Kallobombus* in the middle of the tree close to *Alpinobombus* and to *Rhodobombus* (member of *Odontobombus*-group). Phenetic analyses based on allozymes detected by electrophoresis place *Kallobombus* at different positions clustered with a group consisting of *Bombus - Melanobombus + Megabombus + Pyrobombus* (in part) (Pekkarinen 1979). Pamilo et al. (1981) places *Kallobombus* close to *Psithyrus* and Pamilo et al. (1987) places *Kallobombus* in a group consisting of *Alpinobombus + Subterraneobombus*. Although it is difficult to argue for a basal position of *Kallobombus* based on morphology, there are traits in morphology and in biology that can be regarded as ancestral – the mandibles in females are rather simple without the structures found in most of the bumblebees, also, the colonies are in the ground and never populous. Only a single species, *B. soroeensis*, without specialised biological traits is known. The results from the DNA-analyses are consistent – all indicate *Kallobombus* as the most basal branch within *Bombus*.

In conclusion, the cuckoo bumblebees, previously placed in the genus *Psithyrus*, constitute a monophyletic clade within the true bumblebees. They are indicated to be most closely related to groups within the *Odontobombus* group, especially *Thoracobombus*. The subgenera *Alpinobombus*, *Bombus*, *Megabombus*, *Thoracobombus*, *Pyrobombus and Melanobombus* are all monophyletic clades. Sister-group relationships are indicated between *Melanobombus* and *Alpigenobombus* and between *Mendacibombus* and *Confusibombus*. In all the analyses *Kallobombus* is indicated to be the sister group to all the investigated bumblebees, including the cuckoo bumblebees.

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Revision of the 'ophrynopine' genera *Argentophrynopus* gen. n., *Guiglia* Benson, *Kulcania* Benson, *Ophrella* Middlekauff, *Ophrynon* Middlekauff, *Ophrynopus* Konow, and *Stirocorsia* Konow (Hymenoptera: Orussidae)

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Vilhelmsen, L. & Smith, D. R.: Revision of the 'ophrynopine' genera *Argentophrynopus* gen. n., *Guiglia* Benson, *Kulcania* Benson, *Ophrella* Middlekauff, *Ophrynon* Middlekauff, *Ophrynopus* Konow, and *Stirocorsia* Konow (Hymenoptera: Orussidae). *Insect Syst. Evol.* 33: 387-420. Copenhagen, December 2002. ISSN 1399-560X.

The genera *Argentophrynopus* Vilhelmsen & Smith, gen. n., *Guiglia* Benson, *Kulcania* Benson, *Ophrella* Middlekauff, *Ophrynon* Middlekauff, *Ophrynopus* Konow, and *Stirocorsia* Konow of the wasp family Orussidae are revised. This group of genera of Orussidae occurs from southern United States to Chile and in Japan, southeastern Asia, and Australia. In total, 27 species are recognized, including *Argentophrynopus enigmus* Vilhelmsen & Smith, sp. n., *A. gauldi* Vilhelmsen & Smith, sp. n., *Guiglia rubicunda* Schmidt, sp. n., *Ophrynopus carinatus* Vilhelmsen & Smith, sp. n., and *Ophrynopus hansoni* Vilhelmsen & Smith, sp. n. *Guiglia queenslandensis coronata* Rayment is considered a new junior synonym of *G. sericatus* (Mocsáry); *G. aureola* Benson is considered a *species inquirenda*. *Kulcania tomentosa* (Middlekauff), comb. n., is transferred from *Ophrynopus*. *Ophrynopus philippinensis* Guiglia is considered a new junior synonym of *Stirocorsia kohli* Konow; *Ophrynella rossi* Yasumatsu, syn. n., and *Oryssus trifasciatus* Cameron, rev. stat., are both considered junior synonyms of *Stirocorsia maculipennis* (Smith). Keys and distribution maps of all recognized species are provided, as well as descriptions of all genera.

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Introduction

The Orussidae is a small family of parasitic wasps, in total about 75 species worldwide (Vilhelmsen unpubl.). Most are uncommon and rarely collected, many species being known from only one or a few specimens. Information about their biology is consequently scarce. However, what evidence there is mostly indicates that they are ectoparasites of wood-boring insect larvae, especially Buprestidae (Coleoptera). The family is unique among the parasitic Hymenoptera in not possessing a wasp-waist, the diagnostic feature of the Apocrita. The Orussidae are the sister group of the Apocrita, the clade comprising the two taxa consistently being the most well supported in recent analyses of basal hymenopteran phylogeny (e.g., Vilhelmsen 2001). The Orussidae have a number of specialized features in the ♀ antennae, forelegs, and ovipositor apparatus unique within the Hymenoptera; these are associated with host detection and oviposition in solid wood (Vilhelmsen et al. 2001). The phylogenetic position and peculiar lifestyle of the family makes further investigation into their systematics and way of life paramount to understanding the evolution of parasitism within Hymenoptera.

In this paper, we treat the systematics of an assemblage of genera that traditionally have been considered to comprise the subfamily Ophrynopinae, the remainder of the species of Orussidae being placed in the Orussinae (Guiglia 1965).